

Science Wars and the need for respect and rigour

Dubious claims that science is a purely social construction are not representative of all 'science studies'. But 'constructivism' should not be dismissed if science wishes to strengthen its role in society.

Ever since the Scientific Revolution of the seventeenth century, scientists have been challenged with a disturbing question: do the fruits of their work provide them with privileged access to 'reality'? Initially, the most persistent questioning came from those who felt that their religious beliefs were under threat. More recently, the baton has passed to sociologists and philosophers who seek to study science as a belief system, using a range of techniques shared by anthropologists and social scientists alike.

The outcomes of these new analyses have not always coincided with the traditional self-image of scientists. This has been particularly true of the conclusions of so-called 'social constructivists' who claim that science is as much the product of a continuous dialogue between scientists as it is of controlled, isolated experimentation. While such views remained contained within relatively limited intellectual and political groups, little attention was paid to them by the mainstream scientific community. But, over the past few years, their influence has appeared to flourish not only in the academic world — including school-teaching — but also in the wider community, where it no longer appears so heretical to equate 'scientific truth' with 'the truth as seen by scientists'.

The backlash has, perhaps, been inevitable. Nor is it surprising that it started in the United States, where 'science studies' has, despite its intellectual roots in Europe, taken on a more institutionalized role through the growth of university departments. Initially the backlash had a strong and admitted political component. The achievement of Alan Sokal, the physicist at New York University who brought the issue to wide public attention with his celebrated hoax last summer in the journal *Social Text*, has been to highlight the extent to which the issues transcend simple political ideologies or motivations and reach to the heart of contemporary ideas about science, truth and reality.

The debates triggered by Sokal's hoax have revealed to a wider audience that there is indeed some shoddy thinking, not to say blatant misrepresentation of the results of scientific research, to be found under the banners of constructivism and postmodernism. This lack of rigour is wholly at odds not only with the intellectual standards of the natural sciences but also with those of scholarship in the humanities. Some non-scientific writers, for example, have appealed to scientific concepts, ranging from relativity theory to natural evolution, to illustrate or legitimize their ideas with a crassness guaranteed to embarrass or anger most readers of this journal. It is only too easy to pick out isolated statements that lack appreciation of scientific realities, such as the extent to which experimental data provide both a framework for and constraint on debate about their significance. And it is hard to find clear evidence that insights from science studies have had a positive effect on the development of scientific knowledge itself.

But it would be wrong to tar all of science studies with the same dismissive brush, or to perceive them as wholly irrelevant to scientific progress. Many working researchers would accept much of

what the constructivists say about the importance of social processes in science, ranging from the influence of fashionable ideas on the design of experiments to the negotiations that take place through the peer-review process. The intellectual world of the scientist is not a clinical, passionless void, but one filled with intense personal and interpersonal feelings. That aspect of research may often have little impact on what eventually becomes accepted as scientific truth. But, as is implied by those who continually urge journalists to write about the 'human face' of science, it remains an essential ingredient of scientific progress.

Increasing public understanding

More significantly, those who have been developing our knowledge of science from this perspective are playing an increasingly important role in mediating the relationship between science and society. In France, many of those engaged in what others call science studies identify themselves as sociologists of innovation, and often participate actively in the painful process of managing technological change. In both Britain and the United States, such individuals are coming to play a key role in debates about the public perception of science-related risks. Similarly, the results of their research have become an integral part of the intellectual pool to which those seeking to assuage public fears of the new genetics are turning for advice and guidance.

Indeed, one of the ironies of the present debate is that the avowed goal of the so-called constructivists is one that, in principle, many of their critics avidly endorse: the increased public understanding of science. It is in nobody's long-term interests that such understanding be uncritical: propagating an idealistic image of science is, in many ways, as dangerous as the purely relativistic image that some (but only some) constructivists seek to impose.

In a welcome development, the public debate sparked by Sokal last summer in the United States appears to have ignited a similar conflagration in Western Europe (see page 381). Equally welcome is the fact that the debate has, perhaps by virtue of public fascination with embarrassment and ridicule, escaped the confines of relatively isolated corners of the academic world and the political left. The stakes on both sides are high. On the one hand, some scientists believe that they are fighting for the intellectual and social credibility of an enterprise that remains essential for human well-being. On the other, many social scientists argue equally convincingly that only a deep understanding of science as a social (as well as intellectual) process will enable us to strengthen the bridge between the worlds of science and politics that is essential if this well-being is to be achieved.

Where public perceptions of science are undermined by slipshod scholarship and misrepresentation, let battle continue. But scientists who reflect at all about the wider significance of their work stand to benefit from a sharpened awareness of the genuine insights that science studies can offer. □



Republican senators promote a doubling of funds for research

[WASHINGTON] Two measures introduced by Republican senators last week would double US government funding for science and biomedical research, one over the next ten years and the other over five. Even though neither goal is likely to be achieved, the proposals are an early sign that the new US Congress may increase its investment in science, even as it struggles to balance the federal budget.

Senator Phil Gramm (Republican, Texas) introduced his National Research Investment Act on 21 January. If passed, the bill would increase federal funding for basic science and medical research from \$32.5 billion in 1997 to \$65 billion in 2007 through steady increases of just over 9 per cent each year.

The bill would benefit the National Science Foundation, the National Institutes of Health (NIH), the National Aeronautics and Space Administration and nine other agencies that conduct research. But it is not specific about how the increase would be divided among these agencies, except to specify that funding for NIH would double to \$25.5 billion within ten years.

The bill calls for funds to be allocated on the basis of peer review. But it rules out

spending on commercial technologies, a move that could well provoke a debate about applied science versus basic science similar to the one that preoccupied the previous Congress.

A Senate resolution introduced the same day by Connie Mack (Republican, Florida) would be even more generous to the NIH, doubling its funding in five years instead of ten. Mack's resolution, however, would not be binding. It merely expresses a "sense of the Senate" that spending on biomedical research should be doubled.

Arlen Specter (Republican, Pennsylvania), who chairs the Senate panel that decides NIH funding appropriations, called that a "lofty goal" and signed on as a co-sponsor of Mack's resolution. But he said he had "grave doubts" that it would be possible. He promised to push for a budget increase of 7.5 per cent — \$950 million — for NIH next year, while warning that even this would be "difficult" and a "long stretch".



Specter: a 'lofty ideal', but is it feasible?

The Senate proposals came as welcome and surprising news for science lobbying groups, who have been hesitating about asking for rises of 6 or 7 per cent (see *Nature* 384, 393; 1996 & 385, 103; 1997). But their pleasure has been tempered by the expectation that President Bill Clinton's 1998 budget (for the year beginning 1 October 1997), to be unveiled early next month, will not include any large increases for science.

Neither Mack nor Gramm — who strongly supports balancing the federal budget — suggest what government spending might be cut to pay for an increase in research funding. Specter, doubtful that the normal appropriations process can provide the extra \$2.5 billion a year needed to double NIH's budget by 2002, could only invite his Senate colleagues to "look toward alternative methods of financing".

Science supporters in and outside Congress are sure to offer suggestions. A new group called Citizens for Public Research and Education Funding, which has its inaugural meeting in Washington this week (see box), proposes a nationwide tax on health-care expenditure to pay for medical research.

Senator John McCain (Republican, Arizona), who chairs the Senate appropriations committee for science, addressed part of the problem last week by introducing a bill (S. 199) to require commercial interests to share the cost of building and operating new federal research facilities intended to benefit their industries.

Even though they lack specific details, the new Republican proposals are significant, given their powerful sponsors. Gramm plays a leading role in Medicare reform, one of the most important issues facing the new Congress, and one that will figure prominently in any plan to balance the budget. Mack chairs the Senate Republican Conference, which helps to shape the party's agenda.

According to some observers in Washington, Senate Republicans see science funding as popular with the voters. Recent remarks by the Senate Majority Leader, Trent Lott (Republican, Mississippi), who takes over this year from the losing presidential candidate, Bob Dole, also raise the hopes of those seeking more funding for NIH. "We're going to look very seriously at changing the priorities and increasing the spending on medical research," said Lott in a speech on 18 January to his Republican colleagues. He returned to the theme at a press conference three days later, saying: "We think medical research has been kind of starved out."

Tony Reichardt

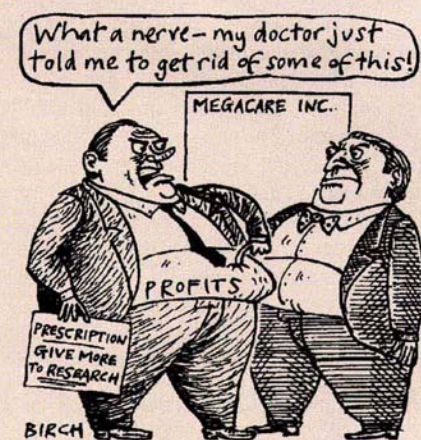
Insurance levy could fund medical centres

[WASHINGTON] A newly formed lobby group is proposing a 2 per cent surcharge on US health-insurance providers to help to fund education and research at academic medical centres that are suffering because of the growth of 'managed care' as the basis of US medical support.

The group of about 30 physicians and scientists, called Citizens for Public Research and Education Funding, was planning to call at its founding meeting on 28 January for national legislation levying the surcharge on all health-care payers, with the proceeds to go to medical research and teaching.

One founding member, George Mandel, a professor of pharmacology at George Washington University Medical School in Washington, DC, and chairman of the National Caucus of Basic Biomedical Science Chairs, backs the surcharge because health-maintenance organizations and insurance companies are "not planning to put any money into research".

Another member, David Pearle, a professor of medicine at Georgetown University, argues that what he calls the "absolute devastation" of research and



teaching at academic medical centres makes it "almost inevitable that something like this [surcharge] is going to happen".

Separately, Senator Daniel Moynihan (Democrat, New York) last week introduced the Medical Education Trust Fund Act. The bill would fund medical education through a 1.5 per cent surcharge on health-insurance premiums, as well as drawing support from Medicare and Medicaid, the government health programmes for the elderly and the poor.

Meredith Wadman

Health scientists strike over cuts at Argentinian labs

[LONDON] Staff at Argentina's largest public health laboratory are taking indefinite strike action in protest at large-scale staff and budget cuts to the country's seven national institutes of health.

Employees at the National Institute for Microbiology are protesting against a 37 per cent cut in the supplies budget, and 100 job losses from a total workforce of 900. Striking staff are occupying the institute's buildings and have vowed to continue their action unless the decision is reversed.

The microbiology institute is the largest of the seven national health institutes which include centres for medical genetics, nutrition and epidemiology. The institute has 370 staff and is responsible for monitoring and controlling infectious diseases, such as the current outbreak of hantavirus in the south of Argentina, which is causing 20 new cases each week, and an outbreak of cholera in the north. Researchers working in disease control are not on strike.

The cuts are part of wide-ranging reforms designed to prune Argentina's bureaucratic public sector. A spokesman says that redundancies have been made largely among non-essential staff. None of the axed scientists, he says, works in disease prevention and control.

But Jeronimo Cello, a researcher at the National Institute for Microbiology, says research scientists make up half of those being made redundant, including many with skills and knowledge not easily found in Argentina. "This is a ridiculous waste of rare scientific talent."

Negotiations between the two sides have so far proved fruitless. The strikers are pinning their hopes on a petition filed in the courts alleging that the government acted illegally by firing its employees. A decision is expected later this week.

Ehsan Masood

Study discloses financial interests behind papers

[WASHINGTON] The debate about whether scientists should declare their financial interest in research published in leading journals has been rekindled by a study of almost 800 original articles published in 1992 by academics from research institutions in Massachusetts.

The reviews, which covered papers appearing in 14 journals of cell and molecular biology and medicine, found that one-third of lead authors had a direct financial interest in the published research.

The journals covered in the study, which was published last month in *Science and Engineering Ethics*, include *Nature*, *Nature Genetics*, *Science*, *The New England Journal of Medicine (NEJM)*, *The Lancet* and the *Proceedings of the National Academy of Sciences (PNAS)*.

Overall, 15.3 per cent of 1,105 lead authors (defined as the first and last authors named) had a financial interest in at least one of the articles. Lead authors of 34 per cent of the 789 papers had a financial interest in the research they were describing.

'Financial interest' was defined as: the author being listed as an inventor in a patent or patent application closely related to the published work (this occurred in 22 per cent of the articles studied); serving on a scientific advisory board of a company developing products related to the author's expertise (20 per cent); or serving as an officer or major shareholder of a company with commercial interests related to the research (7 per cent).

Consultancies, personal financial holdings and honoraria were excluded, on the grounds that such links could not be adequately documented.

"If we're serious about making financial interest a part of the ethics of science, then we have to address the magnitude of the commercial ties that scientists have with their

work," says Sheldon Krimsky, professor of urban and environmental policy at Tufts University in Medford, Massachusetts, and the lead author of the new study.

Krimsky suggests extending a 1995 policy that now applies to scientists seeking federal grant money in the US Public Health Service, which includes the National Institutes of Health. This requires grant applicants to disclose "significant" financial interests of more than \$10,000 that "reasonably appear to be affected" by the proposed research. The National Science Foundation has a similar policy.

Krimsky argues that such disclosure should also be required of all federally funded scientists seeking to publish papers in scientific journals. At present, some journals require authors to disclose any commercial associations that might pose a conflict of interest. Others require disclosure to the journal's editors when authors submit articles. Neither policy is universally accepted.

Some scientists protest that what they describe as an implicit suggestion of wrongdoing by authors who have financial interests in their subjects risks creating an atmosphere of 'scientific McCarthyism'. "It's crucial in science to judge a work by its merit and not by the author or authors' alleged biases," says Kenneth Rothman, a professor of public health at Boston University and editor of *Epidemiology*.

Rothman attacked the mandatory disclosure policies of some journals in a 1993 essay in the *Journal of the American Medical Association (JAMA)* (269, 2782-2784; 1993). The growing aversion of editors to publishing work by authors with financial interests in their subjects "diminishes scientific interchange, in the long run reduces objectivity, and harms scientific method," he says.

But others say that the study has thrown useful light on an important trend. George Lundberg, the editor of *JAMA*, says that financial conflicts of interest among scientific authors are "widespread" but that readers are largely unaware of them "because so many editors are not following what we consider to be good editorial practices of requiring and publishing financial disclosures routinely". (*JAMA*, which was not part of the study, requires financial disclosure by its authors.)

Among the 14 journals studied, four require financial disclosure by authors: *Science*, *NEJM*, *The Lancet* and *PNAS*. But in 1992 — the year of the study — only *NEJM* required disclosure. *Science* introduced its disclosure policy in July 1992, *The Lancet* in 1994 and *PNAS* in 1996.

Meredith Wadman

First German BSE case worries consumers

[PARIS] Consumer confidence in German beef suffered a psychological blow last week with the announcement that for the first time a cow born in Germany had contracted bovine spongiform encephalopathy (BSE).

Although Germany has already reported four cases of BSE, these occurred in cows that had been imported.

The announcement will comfort sceptics who have long argued that many European countries have under-reported cases of BSE. They argue that UK exports of contaminated meat and bone meal and breeding cows should have resulted in several thousand

cases of BSE on the continent, rather than the 50 or so that have been declared, citing as one reason the lack of compensation schemes to encourage farmers to declare cases (see *Nature* 382, 4; 1996).

Meanwhile, German officials say there is as yet no reason to suspect that a 41-year-old woman who died of a neurodegenerative disease was suffering from the new form of Creutzfeldt-Jakob disease (CJD) that has been proven to have caused the death of 14 people in the UK and two in France, and that is suspected to be due to consumption of beef products contaminated with BSE. □

UCSF settles lawsuit over research costs

[SAN DIEGO] In an unprecedented decision, a private US foundation will formally agree this week to pay \$25 million to settle a lawsuit over allegations concerning a long-running scheme under which the University of California at San Francisco (UCSF) claims to have been cheated out of income from research funds.

The foundation's founder and chief executive is Dennis T. Mangano, a physician who until last year was a vice-chairman of anaesthesia at UCSF. He is facing charges from the university of violating a large number of academic policies, including intentionally misappropriating the research and intellectual property of colleagues.

The main legal charge against Mangano, in a suit filed by another UCSF faculty member, is that he used his Ischemia Research (IREF) and Education Foundation to secure at least \$50 million in grants from drug companies in California and Europe without paying the university the full costs of using its facilities. Court records state that UCSF received only \$2 million from the grants.

Non-profit foundations controlled by university professors have caused increasing concern at the University of California's five medical campuses, which have been hit hard by budgetary restraints. The foundations developed into multi-million-dollar enterprises as pharmaceutical companies increased their funding of clinical trials through the organizations, which were often able to make free use of university facilities.

Mangano, who insists that he did nothing wrong, says that university officials were aware of his foundation's activities, which were designed to capitalize on the combined efforts of associated researchers at 160 institutions in the United States and elsewhere.

Arguing that the board of the foundation has agreed to settle the lawsuit without his involvement, Mangano says: "The public should look at the bottom line — which is that money [intended] to be used for finding a cure for heart attack and stroke is now being diverted. I believe this sets a very bad precedent for non-profit foundations."

Although the lawsuit is due to be officially settled tomorrow (31 January) after more than two years of litigation, the UCSF allegations against Mangano for his academic behaviour remain unresolved — a fact that has troubled some of those familiar with the alleged offences.

The concern arises from the fact that, although the allegations of academic misconduct were made nearly three years ago, UCSF officials have yet to complete their internal investigation, or to take action over the allegations of Mangano's abuse of professors and students, and of the collegial process by which research information is

shared. Such inaction has raised questions about how the university deals with allegations against revenue-producing clinical researchers at its hospitals facing financial difficulties.

The importance of such questions is growing as UCSF and Stanford University merge their hospital facilities into a single, private organization, whose operations will be largely shielded from public scrutiny. One widespread fear is that the publicly owned UCSF facility and its employees could be abused more easily after the merger.

UCSF officials decline to comment on either the Mangano lawsuit or the academic inquiry. It was Charles A. Richardson, an assistant adjunct professor at the university, who filed the lawsuit in a state court under a seldom-used California statute that allows a 'whistleblower' to sue to recover funds for a stated institution — in this case UCSF.

Under the law, most of the recovered funds go to the state institution, but the whistleblower and his or her attorney also can share the award. The California law is similar to the federal whistleblower law, which has been used to recover money for the National Institutes of Health after false claims in grant applications. But legal authorities say this is the first time the California law has been used in such a case, whose \$25-million settlement is unprecedented in American academic history.

Under the terms of the settlement filed in San Francisco Superior Court, UCSF will receive \$19.2 million from the foundation, Richardson will — in line with federal legislation allocating a percentage of money recovered as a reward to whistleblowers — get \$1.5 million, and his attorney's fees and costs of \$4.3 million will be paid.

Mangano, who remains a UCSF professor and whose anaesthesia practice is based at the Veterans Administration Medical Center in San Francisco, remains opposed to the settlement. "These dollars were to perform research on high-risk surgical patients," he says. "I find it very discouraging that powerful individuals have found a way to [turn] the money [to a] personal gain."

Richardson declines to comment on the settlement (as do three other UCSF researchers with whom Richardson filed the academic complaint against Mangano in June 1994). But sources at the university say he hopes the university will use the \$19.2 million to help restore the fortunes of those whose careers have been adversely affected by the actions of Mangano and his foundation.

In the academic complaint, the four researchers alleged that Mangano significantly interfered with "free inquiry and exchange of ideas", that he "prevented the extension of knowledge by faculty, fellow and students", used his "position of power" to "cause harm to a student for professional and personal reasons" and "harassed and intimidated" UCSF researchers so they could not carry out their university jobs.

The four researchers requested an audit of Mangano's activities, but the audit — largely completed several months ago — has not yet been released by UCSF authorities, who say it may be available in a month.

Commenting on how Mangano and his foundation operated, one former UCSF official says privately: "There has never been anything like this in the history of higher education. This was full-blown [use of] the university's name and pulling in millions of dollars" with little regard to the impact on the university.

Rex Dalton

UK's top research groups win extra funds

[LONDON] The Higher Education Funding Council for England, which distributes government funding to universities, announced last week that it is introducing a new method for allocating research money which will ensure that the highest-rated universities will receive a greater proportion of total funds.

The money received by universities is based directly on the scores of individual departments in the recent Research Assessment Exercise (RAE) (see *Nature* 385, 3; 1997). In addition to increasing the funding gap between departments reaching different levels of assessment, new cost ratings to be used to weight the initial distribution of funds between subjects will bring relatively more money to life sciences and physics and less to pure mathematics.

The funding council will share out about £630 million (US\$1.02 billion) next month between 136 institutions, on the basis of the total quality of research in each university's departments. RAE grades have been converted into a 'funding factor', which starts at 1 for grade 3b and increases by 50 per cent each step to grade 5, with a 20 per cent premium for grade 5* — for a factor of 4.05.

Even with this special treatment, 5* departments will receive little more than did all departments awarded 5 in academic year 1996–97 as a result of an increase in overall research performance by British universities. Departments that received a score of 1 or 2 will receive no government funds for research, although they are free to raise funds elsewhere, or to transfer them from their teaching budgets.

Claire O'Brien

French museum 'in decay' fights for its life

[PARIS] Decades of neglect by successive governments have allowed France's National Museum of Natural History in Paris to fall into a dangerous state of decay, according to a report by a government advisory panel.

A serious fire last August at the museum, for example, came within metres of the world's largest herbarium, a tinderbox of 8 million specimens. Had the herbarium been destroyed it would have been an "international catastrophe", says Philippe Taquet, head of the palaeontology department and a former director of the museum.

A crisis has been reached, warns the 270-page report by the CNE, an independent advisory committee set up in 1984 to evaluate public scientific and cultural agencies. The government has now agreed to carry out a detailed audit of the museum's needs before the summer, in what it describes as a first step towards a comprehensive renovation programme.

The report points out that much of the museum, which houses one of the world's leading natural history collections, fails to meet minimum safety requirements. Apart from fire, staff are at risk from such hazards as electrocution, dangerous roofs and poorly stored chemicals.

The herbarium's operating budget is "zero francs, zero centimes", points out one researcher, while its electrical system, like that of much of the museum, is antiquated and still operates at 110 volts.

Similarly, a notice pinned to the wall of the palaeontology department casually informs researchers that no outgoing mail will be sent until the money needed to buy stamps arrives from the 1997 budget, which officially amounts to around FF250 million (US\$45 million).

The origin of the problem, according to the review panel, is that the museum has been one of the lowest spending priorities of its parent body, the education ministry.

Rectifying matters "is a question of political will", says Henri De Lumley, the museum's director. He adds that "the state needs to design a rolling plan of renovation over 15 to 20 years". But he is under no illusion that the education ministry alone will be able to come up with the funds needed, and says that an emergency injection of funds will be needed from the state.

This hint seems aimed at President Jacques Chirac. The recent FF500-million renovation of the museum's Great Hall of Evolution was not funded by the education ministry, but on the personal initiative of Chirac's predecessor, François Mitterrand (see *Nature* 362, 280; 1993).

The report also criticizes the museum itself for paying insufficient attention to the upkeep of its collections. These include 7 kilometres of 'carrots' from deep ocean drilling, 2 million fossils, 150 million insects, and 1.5 million vertebrates, including 1 million fish, 200,000 amphibians and reptiles, and 272,000 birds and mammals.

Part of the problem is that the upkeep of the collections is left to researchers. They are under such pressure to publish papers that they have little time to devote to the collections on which they rely, says De Lumley.

He points out that many of the museum's researchers are employees of the Centre National de la Recherche Scientifique (CNRS) or other research organizations, and that such agencies evaluate performance in terms of research and not on the basis of contributions made to maintaining collections or creating exhibitions.

Resolving the problems requires the renovation of storage facilities for the collections, and the creation of a corps of dedicated curators, according to the report. De Lumley supports both proposals, but argues that they can be accomplished only if the museum's budgets for collections are increased to a "realistic" level.

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Skeleton staff? Extra funding, as the Great Hall of Evolution received, would help the museum.

The broad thrust of the report is that the museum needs to refocus its activities on its three specific missions — conservation of collections, research, and informing the public — and in a way that promotes understanding of biodiversity, evolution and the environment.

It also criticizes the complexity of the accounting systems used by the museum, claiming that this makes it difficult to work out what share of the budget is devoted to each of the museum's missions. The Cours des Comptes is completing an audit of the museum, which is expected to be released next month.

The report argues that although many of the museum's fundamental research laboratories are internationally renowned, much of the work is essentially medical, or duplicates that done elsewhere. This represents a drain on the museum's scant resources that it cannot afford, says the report, arguing that money would be better spent by focusing on research that only the museum can do.

Some of the criticism can be traced to resentment by traditional systematists at the high costs of such facilities as DNA sequencing laboratories, when they have no funds to maintain their collections.

But Taquet admits that the museum has suffered from an overemphasis on molecular biology over the past two decades, and that systematics is enjoying a comeback. "I agree that the museum cannot be in all areas of research and that we need to focus on those areas that are central to our mission."

De Lumley has begun streamlining the museum's research activities by regrouping its laboratories within interdisciplinary "institutes", such as "ecology and management of biodiversity".

Declan Butler

Pig heart transplant surgeon held in jail

[NEW DELHI] An Indian heart surgeon who last month carried out an unsuccessful attempt to transplant a pig's heart into a 32-year-old man is being held in custody in Guwahati, the capital of Assam state.

Bail has been denied to the surgeon, Dhaniram Baruah, and two colleagues — C. J. James and Jonathan Ho of the Prince of Wales Medical Institute in Hong Kong — who assisted in the operation. The patient died soon afterwards.

The three have been held in jail in Guwahati since 10 January after being charged with violating the Organ Transplant Act of 1994. Their arrests followed

complaints to the police from relatives of the patient that the death took place "under mysterious circumstances".

Baruah, who runs a heart institute in Guwahati, is believed to have carried out the transplant to generate publicity. His arguments that the law did not apply to Assam state, and that he had the patient's consent for the surgery, were both rejected by the Indian court. The court also rejected a separate bail application for Ho filed by his wife. The case will be heard next month. If convicted, the doctors could receive a fine of up to Rs10,000 (US\$300) and a jail term of five years.

K. S. Jayaraman

Japan's demography poses questions for old and young alike

[TOKYO] Japan faces even more rapid ageing and decline in numbers of its population than previously estimated, according to a study released last week by the Ministry of Health and Welfare's Institute of Population and Social Security Research. The gloomy projections do not bode well for the country, which is already confronted with an unfavourable economic future.

The institute predicts that Japan's population will peak at just under 128 million in 2007, four years earlier than estimated in a survey in 1992, and will decline steadily thereafter to 100 million by 2050. This latest estimate is based on demographic projections from an analysis of the 1995 population census, the birth rate and life expectancy.

The population of people over 65 will rise rapidly to 32 million by 2015, constituting a quarter of the population (compared with 15 per cent at present), while the working-age population, aged between 15 and 64, that has to support them, will fall from 87 to 76 million. The working population will continue a steady decline to only 55 million — barely half the population — in 2050.

In 1994, Japan was eleventh in the league of developed nations with large elderly populations; Sweden topped the list, with 17.5 per cent of the population over 65. But by 2015 Japan will have the world's largest proportion of elderly people.

The pessimistic projections are driven by a declining birth rate, which dropped to a record low of 1.42 per woman in 1995, and high life expectancy, which stood at 83.0 years for women and 76.6 years for men in 1994. The institute projects that life expectancy in Japan will rise to 86.5 for women and 79.4 for men by 2050 because of improved health care, while the birth rate will continue to decline to 1.38 in 2000 and then rise gradually to 1.60 by 2022. The 1992 survey had predicted a recovery in the birth rate from 1995 onwards, but that has proved wrong.

The projections of a more rapidly ageing society combined with a more rapid decline in the working-age population will put an enormous strain on the national health-insurance system, which is already destined to plummet into the red unless there are major reforms requiring patients and working people to pay more.

Analysts say that the latest projections are expected to drive the pharmaceutical industry to invest even more heavily than it is already doing in the development of drugs to treat the diseases of old age, such as Alzheimer's disease.

David Swinbanks

Canada and France fall out over the risks of asbestos

[PARIS AND MONTREAL] Sharp differences are emerging between the federal government of Canada and Canadian and other health experts over the government's decision to ask France to rethink a recent ban on asbestos. Canada is the world's largest exporter of the material.

The government argues that a scientific report by an international panel set up under the auspices of the Royal Society of Canada (RSC) undermines the findings of a report by an expert panel of INSERM, France's national biomedical research agency, that was released on the eve of the ban last July.

Jean Chrétien, Canada's prime minister, said in Paris during a state visit to France last week that the Canadian report would "convince" France to reconsider its ban and show that "there were ways to use asbestos safely".

A statement from the Canadian government said that INSERM had "overestimated the real threat of asbestos to the French population". But some members of the RSC panel that produced the report have distanced themselves from the political use being made of it.

Robert Haynes, president of the RSC, says that the setting-up of expert panels "at arm's length" from government is a vital role of the society. But he says that the panel's conclusions say nothing about the validity of the French ban. The RSC itself "is absolutely not putting any political interpretation" on the study, he says.

Haynes is not surprised by the political interpretation of the study. "It is inevitable in such controversial issues that politicians will read whatever they want into it," he says. "Even the devil can quote scripture to his own ends."

Enzo Merler, an Italian scientist on the panel who works at the International Centre for Cancer Research in Lyon, France, says it is "wrong" to use the panel's findings to argue for a lifting of the ban. He argues that Canada commissioned the report to support "a position against France", adding that he personally feels the INSERM report was of "exceptional quality, exactitude and comprehensiveness".

Merler says: "Risk estimates are just one input into social, economic and political judgements". He argues that decisions on whether to ban asbestos depend on many other factors such as economic feasibility, and the availability of safe substitutes.

France was right to ban asbestos, argues Merler. He says that it is difficult to apply safety measures in areas such as building maintenance, where workers are exposed to low levels of asbestos."

The RSC panel also points out in its report that it was asked for a critical analysis of the INSERM report, and it is therefore not surprising that much of its comments are negative. F. Kenneth Hare, chairman of the RSC panel and a professor emeritus at the University of Toronto, says for example, that "although the French unquestionably have a problem, it isn't quite as grave as the [INSERM] report says".

Hare emphasizes that the panel agreed with much of the INSERM report, including its conclusion that all forms of asbestos are carcinogenic. Hare says that the Quebec government, in particular, "has got a problem on its hands". Asbestos represents 7 per cent of Quebec's exports, and in 1994 the province accounted for 524,000 of the 531,000 tons — worth some C\$233 million (US\$179 million) — produced in Canada as a whole. Although Canada exports just 6 per cent of this to France, it is concerned that other countries may follow the French move. Chrétien has said that he intends to fight the ban at the World Trade Organization.

The RSC study has itself come under criticism. The fact that it was produced within a few weeks is criticized by three of the four external academics to whom it was sent for peer review. One pointed out that corresponding studies elsewhere often take years. "Whoever decided on these constraints [of time] cannot have had any idea of the complexity of the questions that had to be addressed," says another, adding that the panel had done a "good job" given the circumstances.

Officials at INSERM are also upset that, although the RSC review has been released to the press, the agency has not yet been formally sent a copy. Marcel Goldberg, one of the rapporteurs of the INSERM report, says this makes it impossible to comment on the panel's criticisms. "It's extraordinarily improper," he says. Haynes replies that the RSC was under contract to deliver its report to Canada Health, but agrees that it "would have been desirable" for the latter to have sent INSERM a copy.

The allegedly surreptitious manner by which the RSC obtained the INSERM report has also created ill-feeling. "I don't know how they got it," says Goldberg, who says no formal request was ever made. Haynes says that he is unaware of how the report was obtained.

Declan Butler & David Spurgeon

ESA plans put solar science out of orbit

[MUNICH] European Solar System scientists, hit hard last year by the failure of Cluster and the Russian Mars 96 mission — are now the biggest losers in the reshuffling of the long-term space science plan of the European Space Agency (ESA).

The plan, known as Horizons 2000, was revised last week by ESA's Space Science Advisory Committee, a group of a dozen independent space scientists, to cope with budgets which are likely to be at least 25 per cent lower than planned for after 2000.

One immediate result has been the decision to drop from the schedule a planned mission to the planet Mercury. The budget cut is also likely to mean that European scientists will play a relatively minor role in the planned international missions to Mars and the Next Generation Space Telescope.

Horizons 2000 was approved by ESA in 1994 as a 20-year space science programme, with launches of major 'cornerstone' missions every three to five years interspersed with medium-size missions. Its goal is to ensure a regular flow of results to each of Europe's space science research communities, including astrophysicists, planetologists and fundamental physicists.

In the optimistic climate of the time, the plan assumed not only index-linked growth in ESA's science budget to the end of the century but also a 5 per cent annual

growth in real terms between 2001 and 2005.

But, late last year, ministers from ESA's 14 member states decided that the growth of the budget of the space science programme should no longer be increased at the rate of inflation between 1996 and 1998. In the absence of further ministerial action, the level budget will automatically continue for two more years and a real increase in budget after 2000 now seems out of the question.

Despite the new budgetary constraints, the advisory committee, whose recommendations have always been accepted by ESA, wants to maintain all the scientific aims of Horizons 2000. This means extending its timetable and being less ambitious in the long term. "Given the uncertainty of future funding", says its chairman Lodewijk Woltjer, an associate of the Observatoire de Haute Provence, southern France, "it no longer made sense to plan missions beyond 2010."

Missions already under way will adhere to their planned schedules. These include Cassini-Huygens, the NASA-led mission to Saturn due for launch in the autumn, a satellite dedicated to X-ray astronomy (1999 launch), Integral, the international gamma-ray astronomy laboratory (2001) and the cometary mission, Rosetta (2003).

The launch schedule for other planned missions will be extended. The cornerstone mission FIRST, the Far Infrared Space Tele-

scope, and the Planck Surveyor, previously known as COBRAS/SAMBA, which will examine the cosmic microwave background, will each be launched with relatively short delays between 2005 and 2007.

An additional medium-sized mission, earmarked for planetary science, will be launched later in the decade. But ESA's commitments now go no further. This means that the cornerstone mission to Mercury, originally due to launch in 2009, is now on hold. So, too, are cornerstone missions dedicated to interferometry and the study of gravitational waves.

The advisory committee says that the advanced technologies required for these missions should continue to be developed in the expectation that they will be rescheduled in the future. Such technologies include ion propulsion, important for more efficient interplanetary travel. But the committee expresses concern that, even with the extended timetable, ESA's science programme will now have little money left to support such development studies.

Nor may there be sufficient money to make a significant contribution to two international programmes judged to be highly important for European space scientists. These are the missions to Mars and the Next Generation Space Telescope, the follow-up to the Hubble Space Telescope.

The committee recommends that the next medium-size mission, to be chosen in the next two or three years, should be at least in part dedicated to a Mars project. It asks the ESA science directorate to set up a task force to identify opportunities for ESA scientists to participate in NASA-led Mars missions, and to decide if use can be made of blueprints for instruments developed in Europe for the ill-fated Mars 96.

"But any participation in a mission to Mars will be disappointingly small," says Woltjer. The committee also asks ESA to set up a task force to follow the development by NASA of plans for the new telescope. It says the task force should identify opportunities for European astronomers to participate, for example by supplying the launcher or instruments, or by trading observing time with that on ESA's FIRST mission.

Steven Beckwith, director of the Max Planck Institute for Astrophysics in Heidelberg, says that the squeezed budgets which have forced the restructuring of the Horizons 2000 programme "will no doubt weaken European space science".

"It seems a shame that just when European space astronomy is coming into its own — and when the United States is investing heavily in new astronomy missions — its support is being cut back," he says.

Alison Abbott

Mission made possible: the miracle mirror

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

[WASHINGTON] A technician places glass over a mould during the casting of the first of two primary mirrors for the Large Binocular Telescope being built at Mount Graham, Arizona. The mirror was cast earlier this month using a new technique that involves pouring blocks of molten glass into a 'honeycomb' mould from a furnace at temperatures of 1,100°C.

The process, known as 'spin casting', took 70 hours and was watched intermittently by 1,700 people. A further 12-week wait is in store before the mirror is cool enough for

inspection; it will then have to be ground and polished. "Only then will we know whether the casting is a success," says Peter A. Strittmatter, director of the Steward Observatory at the University of Arizona.

The Large Binocular Telescope will consist of two primary mirrors, each 8.4 metres in diameter. Once completed, the Mount Graham instrument will have a resolving power which corresponds to that of a single 22.8-metre telescope, and light-gathering power equivalent to that of a single 11.8-metre instrument. □

The 'Sokal affair' takes transatlantic turn

[LONDON] A dispute that has been simmering since last summer in the United States over the validity of 'postmodernist' ideas about the nature of scientific knowledge has finally reached the point where many such ideas originated — the banks of the river Seine in Paris.

Over the past month, the newspaper *Le Monde* has been running a series of articles triggered by an account of the widely publicized hoax perpetrated last year by Alan Sokal, a theoretical physicist at New York University, on the journal *Social Text*.

The hoax took the form of an article submitted to and accepted by the journal. It purported to demonstrate the social and political origins of ideas in quantum mechanics — but in fact was fabricated out of miscellaneous (but accurate) quotations from prominent postmodern writers and dubious statements of scientific 'fact'.

Sokal's article has added fuel to a conflict that has been growing in recent years between scientists who argue that science is based on empirical fact, and sociologists of science who argue that much of scientific knowledge is 'constructed' out of debates between researchers (see, for example, *Nature* 375, 439; 1995).

In the United States, the hoax article and its implications — namely that sociologists of science have little regard for empirical truth and are more interested in intellectual fashions — has set off a wide debate on university campuses. "The reaction has been a factor of ten bigger than I expected," says Sokal. "And it is not letting up."

Until now, the response in Europe has been relatively muted, even though many of the writers quoted tend to be European, usually either British or French. The main reaction has been a defence of European academics whose work and US colleagues have come under attack.

Petitions, postmodernism and politics

Last October, for example, many of those attending a joint meeting of the US-based Society for Social Studies in Science and the European Association for Studies of Science and Technology, held in Bielefeld in Germany, signed a petition protesting that some of the recent US criticism of work by sociologists of science could, in Europe, be regarded as potentially defamatory.

But the recent series of articles in *Le Monde*, widely regarded as the main public forum for both intellectual and political debate in France, as well as coverage in French publications *Libération* and *Le Nouvel Observateur*, indicate that the issue is now heating up in Europe too.

Further evidence comes from the fact that an article by Paul Boghassian, a philosopher

also at New York University, attacking post-modernist views of science, which appeared in the *Times Literary Supplement* in December, has already been published in *Die Zeit*, one of Germany's leading newspapers.

One of Sokal's strongest supporters is Jean Bricmont, a theoretical physicist at the University of Louvain in Belgium. He is writing a book with Sokal on what both argue is the frequent misuse of scientific concepts by prominent — and mainly French — intellectual figures ranging from the psychoanalyst Lacan to Bruno Latour, an influential sociologist of science.

When is a fact is not fact?

Bricmont wrote in his contribution to the debate in *Le Monde* that such allusions tended to be "at best totally arbitrary and at worse erroneous". He says he is keen to see a reinstatement of ideas about science based on empiricism and the analytical philosophy of individuals such as the mathematician Bertrand Russell, rather than those of German idealists such as the philosopher Martin Heidegger.

He says he is concerned at a growing tendency to see ideas in socially relative terms, criticizing, for example, official guidelines on epistemology used by high-school teachers in Belgium for stating that a fact is not an empirical truth, but "something that every one agrees upon".

Like Sokal, Bricmont says that he has been surprised by the level of interest he has stirred up. "I seem to have put my finger on something bigger than I realized," he says.

But some of those under attack, having initially held back from the fray on the grounds that the debate was primarily based on issues internal to the United States, are now fighting back, arguing that it is



Sokal: hoax article set off enormous debate.

their critics who have an idealistic — and increasingly outdated — vision of science and its role in contemporary culture.

Last week, for example, Latour, who teaches the sociology of innovation at the Ecole Supérieure des Mines in Paris, one of France's so-called 'grandes écoles', complained in *Le Monde* that he and fellow sociologists were being treated as "drug peddlers" who were corrupting the minds of American youth.

In fact, says Latour, one of his main concerns has been to demonstrate how modern society — as reflected in the public response to concerns about bovine spongiform encephalopathy ('mad cow disease') — is transforming itself from a culture "based

on Science, with a capital S", to one based on research more broadly, including the social sciences.

He writes: "In place of an autonomous and detached science, whose absolute knowledge allows us to extinguish the fires of political passions and subjectivity, we are entering a new era in which scientific controversy becomes part of political controversy."

The latest salvo in the French debate comes from Sokal himself. In a response due to be published this week, Sokal repeats his claim that every scientist is aware that, although scientific knowledge is always partial and subject to revision, "that does not prevent it from being objective".

Sokal eschews charges of chauvinism, saying that his target is not — as some have suggested — French intellectuals as such, but "certain intellectuals who happen to live in France". He also dismisses the criticism that his concern about the growing influence of growth of 'constructivist' ideas about science reflects worries about a decline in both funding for physics and the social status with the end of the Cold War.

Differences in culture and education

But Latour, too, who makes both claims, has his supporters — and not just in France. Simon Schaffer, a lecturer in the history and philosophy of science at the University of Cambridge, points to the irony that Latour and others are trying to develop the public understanding of science that, in other contexts, Sokal and others argue is essential if they are to retain respect.

Schaffer also points to the different cultural environments, partly a product of different educational traditions, in which French and American scientists operate. "In France, everyone believes that the sciences are self-validating, and that the social sciences refer to a world that exists outside themselves," he says.

In contrast, he argues, the empiricism that tends to dominate the Anglo-American approach to science means that "no-one in the scientific community sees themselves as an epistemologist or a constructivist".

With Europe facing important issues concerning the relationship between science and politics — ranging from the likely science policy of the British Labour party if it wins the imminent general election, to the squeeze by Germany on international spending on particle physics — the public debate set alight by Sokal appears unlikely to die down rapidly.

David Dickson

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REASONS

Russell: pursued an
analytical approach.

German Greens seek increased support for basic science

[MUNICH] In a significant reversal of policy, the German Green Party has issued a report actively backing research and development, and criticizing Germany's ruling coalition of Christian Democrats and Free Democrats for what it calls "the country's failure in technological innovation".

Since the party's foundation in the early 1980s, it has maintained a sceptical attitude towards science. "However, we now accept the basic importance of science and technology to society," says Manuel Kiper, spokesman for the party's parliamentary group, and a co-author of the new policy document.

The document calls for a reorientation of the goals of research towards sustainable development and social responsibility, and says that basic research should be given higher priority than applied research. It criticizes research minister Jürgen Rüttgers for overemphasizing industrial research aimed at short-term economic gain. But the party has not reversed its stand on genetic engineering, to which in principle it remains opposed. This issue is to be tackled in a series of meetings being organized throughout this year by the Greens.

Physicists share award from Faisal foundation

[LONDON] Saudi Arabia's King Faisal Foundation has announced its International Prizewinners in Science and Medicine. Physicists Carl Wieman and Eric Cornell of the University of Colorado and the US National Institute for Standards and Technology share the US\$200,000 science award for achieving a Bose-Einstein condensate using a record temperature of 170 nanokelvins.

The medicine prize is shared by James Gusella of Harvard Medical School for identifying the *Huntingtin* gene that is mutated in Huntington's disease, and Colin Masters of the University of Melbourne, Australia, and Konrad Beyreuther of the University of Heidelberg, Germany, for their work on the abnormal amyloid deposits in Alzheimer's disease.

Gulf troops at munitions dump 'suffered more'

[WASHINGTON] Contrary to earlier press reports, Gulf War veterans who helped demolish an Iraqi munitions dump where they may have been exposed to nerve gas have reported joint and muscle pain at a higher rate than other veterans in a government register, a top official at the

Department of Veterans Affairs told a congressional committee last week.

Kenneth Kizer, under-secretary for health at the Department of Veterans Affairs, reported the finding in testimony to the House Committee on Government Reform and Oversight's subcommittee on human resources and intergovernmental relations. An analysis of 1,978 troops within 50 kilometres of an Iraqi munitions dump destroyed by US troops in March 1991 showed that 28.4 per cent of those 81 troops directly involved in its destruction have since reported musculoskeletal pain. This figure compared to 18.4 per cent of the entire group which was within 50 kilometres.

Italian research institutes win collaborative grants

[ROME] Four research institutes in northern Italy have been awarded the first of a series of collaborative research awards granted by the new Giovanni Armenise-Harvard Foundation. Each institute will receive US\$200,000 a year for the next three years. The winning institutes are DIBIT, the basic biomedical research branch of the San Raffaele Scientific Institute of Milan, the European Institute of Oncology, also in Milan, the University of Padova, and Turin University's Institute for Cancer Research.

The foundation was established last year

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Peer reviewers could do much better

Sir — When peers, as in peer review, are not conferencing, site visiting, sitting on study sections or other committees, consulting and even, heaven forbid, vacationing, what are they doing? Word processing, in the form of grant applications, manuscripts, reviews, reports, more reports, letters, faxes and e-mails (the worst). We have in effect become short-story writers. The problem is that good short-story writing is very difficult. It takes time and thought. Yet time is in short supply. Thought too. Do peers have time for proper reviewing, and what is the quality of peers' reviews?

The answer to the first question is precious little. How little time do referees spend on reviewing a manuscript? I've heard of 30 minutes. Although the straightforward can be readily despatched, reviewing is most pertinent when a manuscript falls in the grey zone. Writing but a handful of thoughtful lines easily consumes 30 minutes. If, in addition to being harassed, the referee is nursing some microbial infection, jet-lagged or reeling from a recent lousy review, what can be expected in terms of quality?

Judging by my own experience in the biological sciences, discussions and frank

gossip, the general conclusion is like the comment at the bottom of my school reports — could do better. And that's the polite version. Let's forget the positive reviews which can be assumed to cause little inconvenience, although they too can surprise. What about the negatives? Rarely does one get constructive comment. Not infrequently a paper is dismissed in as little as eight lines or even less. And this after 6–8 weeks. Then there is the backhanded review which compliments the authors on a good piece of work only to say that it is not suitable for, say, *Nature*. Alternatively, when more than a couple of paragraphs are proffered, the referee often betrays either a superficial reading of the text or else a lack of familiarity with the subject. Not infrequently the answer to a criticism can be found in the manuscript. Importantly, reviewing often stigmatizes discussion as speculation. When so few pieces of a jigsaw puzzle are available, how else can connections be made if not by speculation? In other words, how frequently does one come across a stimulating discussion? Note that all of the above examples are accepted by harassed (?) editors.

It is patently clear that reviewing is not a

peer's priority. Is not the publication of so many mediocre papers and the surprising proportion that go unreferenced, even by their authors, evidence of this? What can be done about this woeful state of affairs?

(1) We need a warts-and-all debate. Can editors be blunt and give us some back-room information? For example, what does an editor do with two vapid eight-line reviews? (2) What about requiring at least one single-spaced page as a criterion for refusal? This runs the double risk of helping the authors and exposing the reviewer. (3) Most importantly, ways must be found to make reviewing a higher priority. A financial incentive is an obvious idea but would kill off a number of journals; come to think of it, that might not be a bad idea. Or contract reviewing — a free subscription for a number of respectable reviews. The journals could then demand the review in a 2–3 week period which would be welcome to all. Or a Monteverdi CD.

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Unitary construction

Sir — The *Système International d'Unités* (SI) has given us unit names that are uninformative, unintuitive, incomprehensible to outsiders and thus hugely attractive to most scientists. One can only admire the SI's consistent push to replace clarity with opacity, for example in the change from 'disintegrations per second' to 'becquerel'. Although biologists have the Michaelis constant and the centimorgan, we seem well behind physicists in accepting SI-like unit names. Here are some ideas.

pauling. One turn of a protein α -helix. ("Do you think 6 paulings is long enough for a transmembrane domain?")

crick. One turn of a DNA helix. ("What happens to transcription when the polymerase site is displaced ± 0.5 cricks?")

watson (or chargaff). One DNA base pair. ("It's a big cDNA, 14 kilowatsons.")

huxley. Unit step of a myosin molecule. ("Professor, how many huxleys does myosin take per ATP hydrolysed?")

koshland. 1 nm of conformational change. ("Hey, look at that. Leu 197 moved 1.2 koshland when glucose was present.")

nüsslein-volhard. A species' complete set of developmental control genes. ("Not bad,

but in *Drosophila* they've already cloned 0.6 nüsslein-volhard.")

levi-montalcini. Concentration of a growth factor giving 50% of the maximum response. ("Wow, the levi-montalcini was only 100 pg per ml!")

varmus (with apologies to Bishop, Weinberg and the rest). 100% transformation of a cell population by an oncogene. ("Uh-oh, Src only gave me 0.1 varmus on 3T3 cells.")

Such unit names should provide many benefits to biologists. They will provoke interminable arguments about which scientists should be memorialized, make it harder for students to learn enough to compete with their mentors, and allow older biologists either to congratulate themselves on learning the latest fashions or to lament the decline of civilization (my own preference). But will biologists, like physicists, find these undoubted benefits to be worth the obfuscation produced by such a clumsy and inappropriate system?

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Not 0 but O

Sir — In your News story "Novel pathogens beat food safety checks" (*Nature* 384, 397; 1996), you designate the strain of *Escherichia coli* 0157:H7. But the first character is wrong. Instead of the numeral 0 (zero), it should be the letter O. Serotypes (serovars) of bacteria are differentiated by the O (*Ohne Hauch*) antigen on the cell envelope (outer membrane) and the H (*Hauch*) antigen on the flagella.

The source of O and H is a paper in the *Wiener klinische Wochenschrift* (30, 1509–1511; 1917) by Edmund Weil (1880–1922) and Arthur Felix (1887–1956), "*Weitere Untersuchungen über das Wesen der Fleckfieberagglutination*" ("Further studies on the nature of typhus agglutination"): "*Der kürzeren Ausdrucksweise wegen nennen wir die typische, hauchförmig wachsende Proteuskolonie die H-Form, die ohne Hauch wachsende die O-Form.*" (Because of the shorter mode of expression we call the typical filmy growing *Proteus* colony the H-form, the one growing without film the O-form.)

Friedrich Katscher

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Ways to surmount the language barrier

Sir — Paul R. Sanberg (*Nature* 384, 608; 1996) has highlighted a further problem caused by the language barrier, when research published in a non-English language publication, for example Japanese, is missed by researchers who do not read the language and do not find the research listed and indexed in relevant databases. There are two possible solutions to this problem.

The first can be exemplified by the database *Aquatic Sciences and Fisheries Abstracts*, which is sponsored by four United Nations (UN) agencies (the Food and Agriculture Organization, the International Oceanographic Commission, the UN Environment Programme and the UN Division for Ocean Affairs and the Law of the Sea) and which receives input from three other international organizations, and from national partners in more than 20 countries. This large partnership base ensures a good coverage of the aquatic sciences literature, each partner covering the literature in its own language.

The second solution can be illustrated by the compilation CD-ROMs made available by the National Information Services Corporation, where many small databases, compiled for national and regional use, are brought together in one 'anthology' database, with duplicate references listed once only.

Both of these solutions can go some way towards making the work of those who publish in the less-accessible languages available to researchers worldwide.

David S. Moulder

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NO prizes

Sir — There is no doubt that the discovery of nitric oxide (NO) as an important biological mediator has had a major impact on many fields of scientific investigation, including study of the cardiovascular and pulmonary systems which is the focus of our practice and research.

The Lasker Prize in the United States was awarded last year to Robert Furchgott and Ferid Murad for work on nitrous oxides in the body. Although it could be argued that prizes are not important and that it is often difficult to make the right choice, we feel strongly that important omissions have been made in the awarding of the prize.

Although there had been some

speculation linking endothelium-derived relaxing factor (EDRF) to nitrogen-containing compounds, the first paper based on solid experimental data and stating categorically that "EDRF is NO" (*Nature* 327, 524–526; 1987) was written by Salvador Moncada and colleagues, and was followed by many other seminal contributions from the same group including the discovery that "NO is synthesized from L-arginine" (*Nature* 333, 664–666; 1988). Papers of similar originality that have made an important contribution have been published by Lou Ignarro. These publications opened the floodgates for the current level of NO research and inspired many scientists and physicians, including us.

Magdi Yacoub

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South Africa

Sir — *Nature* and its South Africa correspondent, Michael Cherry, are to be congratulated on the Briefing on science in South Africa (384, 11–15; 1996). In many ways the country is unique; indeed, it can be described as two countries in one. In 1992, the country's white community, considered alone, was placed 34 out of a total of 175 countries on the Human Development Index in terms of average life expectancy, educational attainment and basic purchasing power; black South Africans were placed at number 125.

A striking aspect of the industrialized South Africa lies in the high level and quality of its scientific and technological capabilities. Your Briefing includes a reference to the way in which international links are helping to overcome years of isolation in science and other fields. But your coverage was restricted to assistance pertaining to bilateral cooperation, and made no mention of the role of multilateral organizations active in science.

Bilateral assistance is certainly important, but it is no substitute for the broadness of vision, ethical considerations and global concerns normally enshrined in multilateral cooperative schemes. It is for these reasons that UNESCO's programme for science is entitled "The sciences in the service of development". Even during the apartheid years, those involved in bilateral arrangements will have noticed the skewed manner in which science was promoted and practised in South Africa.

UNESCO is helping the South African government to carry out a comprehensive evaluation of academic development programmes in science and technology,

with special reference to the historically disadvantaged institutions. This is expected to recommend how to remedy the unbalanced patterns of access to science, engineering and technology disciplines of disadvantaged students in the short and medium term.

South Africa is also increasing its international scientific links. It has, for example, recently joined the Intergovernmental Hydrological programme, and is preparing to become a member of the Man and Biosphere programme. Membership of these programmes will allow it to tap into intellectual and financial resources for the conduct of scientific research and the exchange of information in key scientific areas related to water resources development, the protection of the environment and sustainable development.

Luis B. Honwana

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Ground rules

Sir — Your News article about repairs to US radiotelescopes (*Nature* 384, 505; 1996) states that the Arecibo Observatory in Puerto Rico is built in the crater of an extinct volcano. In fact, the observatory is located in a sinkhole (a collapsed cave chamber). The Arecibo area has typical karst topography and is riddled with caves. The nearby Tres Pueblos sinkhole is the entrance to the Camuy Caves Park.

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Stone Age confusion

Sir — A recent News article (*Nature* 384, 605; 1996) referred to a taxonomical analysis that "proved that a popular television figure, Barney from the Flintstones, is not a dinosaur after all". Any child knows that Barney from the Flintstones is a Stone Age man, close friend of Fred Flintstone, and lives with dinosaurs despite all the fossil evidence to the contrary. However, Barney, who hosts a children's show on the US Public Broadcasting System, is undoubtedly a dinosaur and pure gold to academics trying to accomplish anything at home while responsible for a toddler.

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Jet propulsion is essential for young stars

David A. Clarke

Many young stars are observed to emit fast-flowing jets of material. A new model of how they are generated confirms the suspicion that episodic jets are an unavoidable consequence of star formation.

Once stars form and nuclear fusion is established, most astrophysicists agree that stellar evolution is well understood¹. However, how stars actually form from gas and dust is quite another story. It is widely held that huge gas clouds somehow fragment, somehow lose enough angular momentum and magnetic energy to collapse, and somehow form planets, binary systems and so on²; but these 'somehows' have had astrophysicists perplexed for several decades. A complicated puzzle such as this can only be assembled one piece at a time, and the paper by Ouyed, Pudritz and Stone on page 409 of this issue³ seems to fix one very important piece in place.

A self-consistent theory of stellar collapse must include several ingredients. It is known that protostars collapse under their own gravity, and in the process lose some 99.99 per cent of their initial angular momentum to allow enough contraction for nuclear fusion to begin². In the earliest stages of stellar formation, it seems that the system rids itself of this unwanted angular momentum by concentrating it into a small fraction of the collapsing matter, turning that matter around, and generating an outflow. The details of the mechanism are complicated, but the principle is simple.

Figure 1 illustrates how the magnetic field is dragged towards the protostar as the cloud collapses. As the rotation rate of the protostar increases (in the same way that figure-skaters increase their spin by drawing in their arms), the differential rotation twists the magnetic field. When the field is twisted beyond a certain angle, the conditions are ripe for the 'bead-on-a-wire' effect to sling out pellets of hot plasma along the magnetic field 'lines'⁴. Indeed, magnetic field can also be dragged out with the flow, further assisting protostellar collapse. The outflow is then collimated by the magnetic field that launches it, generating a spinning, supersonic stream of plasma that astrophysicists call a jet. Jets from young stellar objects are a few light years⁵ long, whereas their big cousins,

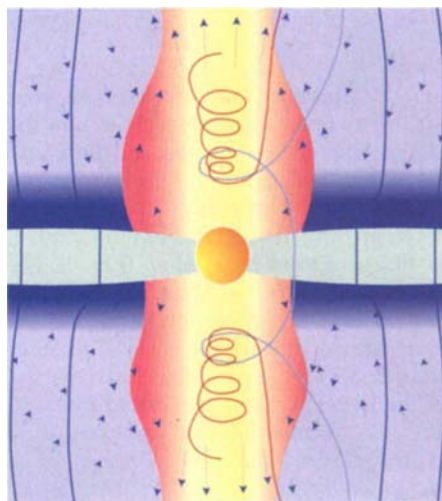


Figure 1 A protostar in its accretion and outflow phase. The yellow protostar at the centre provides the deep gravitational potential well that drives the process. Matter from a gas cloud (blue) falls in towards an accretion disk (green) that orbits the star, dragging magnetic field with it. (Velocity vectors show the speed and direction of the flow; the magnetic field lines have been given different colours for clarity.) Rotation of the gas cloud twists the field lines and provides some centrifugal support, which is depleted by material flung out along magnetic field lines. Much of this outflow is collimated by the magnetic field into two jets (red) launched along the rotation axis of the cloud.

launched from the cores of young galaxies, measure on the order of a million light years⁶. In this picture, collapse and outflow are inexorably coupled by angular momentum and magnetism.

Ouyed *et al.*³ present a solution to a vital piece of the problem of star formation: how are jets launched? Once launched, their propagation is fairly well understood, both computationally⁷ and in the laboratory⁸, but the mechanism by which jets are launched has eluded jet aficionados for some time. Ouyed and colleagues³ find that by treating the accretion disk of the young star as a given boundary condition, they are able to avoid many of the computational

problems that haunted previous investigators⁹.

When the magnetic field threading the disk is sheared by the differential rotation, what seems to be a universal mechanism launches jets whose power, mass flux and variability are consistent with observations of jets in young stellar objects. The model may also hold for jets launched from galaxies and quasars, although Ouyed and colleagues' non-relativistic calculations don't specifically include the realm of extragalactic jets.

Their model is simple and elegant. The magnetic field is twisted until it reaches the critical opening angle — the angle relative to the plane of the disk that gives it enough leverage to launch an outflow. Further twisting increases the magnetic pressure within the jet, forcing it to expand radially (perpendicular to the direction of the outflow). The momentum of expansion causes the jet to overshoot its equilibrium radius, however, and the excess ambient pressure causes the jet to collapse again towards its axis (producing the bulge and waist in the jets of Fig. 1). As it collapses, it spins faster and hits a 'centrifugal barrier', which causes it to expand again.

This oscillation survives for a few periods and comprises what Ouyed *et al.* call the "knot-generating region", in which the jet velocity is necessarily variable. As the jet propagates, the velocity variations steepen to form shocks, which can dissociate the continuous jet into a series of clumps¹⁰. Evidently, this mechanism for launching jets includes a natural explanation for the clumpy nature of many observed protostellar jets⁵.

The simplicity and universality of this mechanism to produce sustained and variable jets set it apart from previous efforts^{9,11}, and is a prime example of the important role played by computational astrophysics. In further computations, the authors have varied their boundary conditions to incorporate every conceivable set of initial condition consistent with the observations, and the results are always the same: a sustained and variable jet (R. Ouyed, personal communication).

Until recently, the importance of the magnetic field has been an unconfirmed assumption of our models. On page 415 of this issue¹², however, Ray *et al.* report powerful evidence for strong magnetic fields in a jet from the protostellar object T Tauri S. This is the first direct evidence that protostellar jets carry an appreciable magnetic field.

A comprehensive theory of star formation still eludes us. Until we are able to model the fragmentation and collapse of a galactic gas cloud into protostellar objects which, via accretion disks and collimated outflows, become dense and hot enough to ignite nuclear fusion, we will not have a complete understanding of this long-standing puzzle.

However, the work of Ouyed, Pudritz and Stone has significantly shortened the stick from which the star-formation-theory carrot is dangling. □

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Cell division

New wrinkles in cytokinesis

Julie A. Theriot and Lisa L. Satterwhite

Once in every cell cycle, the mother cell splits itself into two daughters, each of which receives its fair share of the mother's contents. To do this, not only must the chromosomes be replicated and split with perfect precision by the mitotic spindle (Fig. 1), but all of the other cell contents — including the plasma membrane, organelles and cytoplasm — must be divided, in the process known as cytokinesis. On page 450 of this issue, Burton and Taylor¹ describe a simple and elegant method by which the very weak traction forces that are generated during cytokinesis can be directly observed. Their results support a model in which cytokinesis occurs through increased contractility at the cell's equator.

Chromosomal division and cytokinesis are coordinated, both temporally and spatially. In animal cells, the position of the mitotic spindle dictates the future position of the cleavage furrow². The microtubules in the spindle and spindle asters are thought to communicate with the cell cortex, to direct the accumulation and alignment of the contractile proteins (actin and myosin) that form the furrow. The mitotic spindle has been the focus of much experimental attention, and many molecules that are involved

in its construction and function have been identified. But less is known about how the cleavage furrow is assembled, and the nature of the signals that connect the spindle and the furrow is still a mystery.

To identify the signals from the spindle, we first need to ascertain where they act. The actomyosin cortex is contractile, generating tension over the whole cell surface. However, late in mitosis the furrow becomes considerably more contractile than the rest of the cortex, causing the cell to pinch in half. The relative increase in contractility of the furrow could arise either by a local increase in contractile activity at the furrow², or by a relaxation of tension elsewhere — particularly at the cell poles³. These two alternative models are known as the 'equatorial-stimulation' and 'polar-relaxation' models, respectively, and they predict that the directive signal from the spindle will act at different sites (Fig. 1).

This is a question about mechanics which is best addressed by mechanical means. Because of their large size and convenient spherical geometry, echinoderm eggs (from sea urchins and sand dollars) have been the target of almost a century of pokes and prods. The most influential work

was started by Raymond Rappaport in the 1960s. Using glass needles and capillary tubes, he found that drastic physical deformation of the cortex of cleaving eggs, or relocation of the mitotic spindle to different sites in the cell², caused the furrow to form at a different location. Mathematical modelling that took into account these distorted cell-geometries tended to favour the equatorial-stimulation model^{4,5}. But these conclusions cannot easily be generalized to smaller, non-spherical cells such as mammalian fibroblasts, and polar relaxation might occur simultaneously⁶.

Burton and Taylor¹ have addressed the question of where, and how, force is generated in the cortex of adherent dividing mammalian fibroblasts. The nifty technique that they have used was originally developed for the study of cell locomotion⁷. Cells exert traction on any solid substrate to which they adhere, although this cannot be detected on rigid substrates such as glass or tissue-culture plastic. More compliant substrates (such as collagen in the body) are deformed and then reshaped by the traction of adherent cells. Fibroblasts cultured on a thin layer of silicon rubber compress the rubber sheet, causing wrinkles to form and radiate out from the site of cell attachment⁷. Deformation of the rubber sheet can be used to measure the amount and direction of the force that is generated by moving cells⁸.

Starting from these principles, Burton and Taylor devised a method to synthesize rubber sheets that can reproducibly be made more compliant by exposure to ultraviolet light. In this way, the very weak traction forces that are generated by dividing cells can be observed. The length of the wrinkles that are formed turns out to be directly proportional to the amount of force applied. This has enabled the amount of force that is generated at the furrow and at the poles of dividing mammalian fibroblasts to be quantified, as live cells are followed through cytokinesis using video-microscopy.

Burton and Taylor found that the traction that is generated at the cell equator — near the presumptive cleavage furrow — increases over time as the cell progresses through mitosis. Traction then drops precipitously, just as the two daughter cells pinch apart. At this point, the daughter cells begin to try to crawl away from each other, gathering wrinkled rubber sheet between them as they do so. The elastic recoil that is produced by the sheet when the daughters finally let go of each other flings the two cells apart.

Traction generated at the cell pole in those rare dividing cells that attach to the rubber sheet at only one end was also directly observed. Here, too, the tension increases slightly (or more to the point, it does not decrease) before plummeting as the cell cleaves. These results are convincing evi-

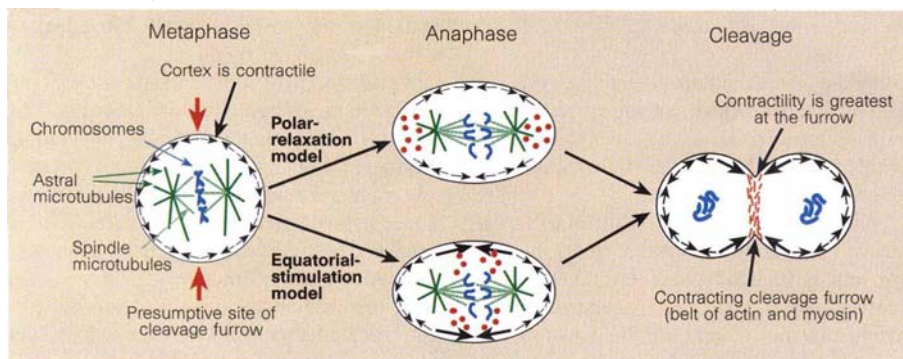


Figure 1 Cytokinesis in spherical cells. Small black arrows represent contractility of the cortex. In the polar-relaxation model, signals (red dots) from the spindle asters cause a relaxation of cortical tension at the cell poles. In the equatorial-stimulation model, the putative signals cause an increase in tension at the cell equator. In either case, net contractility is greatest at the equator, resulting in contraction of the cleavage furrow.

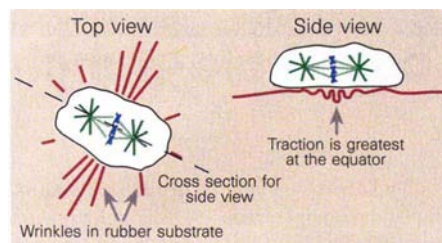


Figure 2 Cytokinesis in fibroblasts attached to a rubber sheet. In the experiments by Burton and Taylor¹, cortical contractility of adherent fibroblasts cultured on a rubber sheet can be detected as the cell traction forms wrinkles in the rubber. Wrinkles are most pronounced at the presumptive site of the cleavage furrow, and no relaxation is observed at the poles, consistent with the equatorial-stimulation model.

dence against the polar-relaxation model, and are consistent with the theory that equatorial stimulation of increased force at the cleavage furrow is necessary for cytokinesis in adherent fibroblasts (Fig. 2).

The molecular identity of the signals from the mitotic spindle that stimulate the formation of the furrow at the correct time and place remains to be discovered, and these signals may not be conserved among different animal species. In echinoderm eggs, the signals seem to be delivered along the astral microtubules that radiate from the spindle poles: a cell containing several spindles will furrow halfway between every pair of asters, even those that are not connected to the same spindle². But this might not be true in mammalian cells, as the spindle microtubules and chromosomes also seem to be necessary to promote furrow construction at the correct site³. Likewise, the exact mechanism by which actomyosin contraction generates force at the furrow remains highly controversial, and this may also differ between echinoderm eggs and mammalian cells¹⁰. Finding the signals, understanding how they are delivered from the spindle and how the cortex transduces the original signals into the huge, complex and still largely enigmatic apparatus of the cleavage furrow, will remain important goals of cell-biological research for years to come. □

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Oceanography

Bottom line for hydrocarbons

Ian R. MacDonald

Submarine gas hydrate, a combination of water and methane, may be the motherlode of hydrocarbon deposits — a pool of organic carbon much larger than global oil reserves, and capable of interacting with the atmosphere to influence climate cycles. But that depends on how to interpret a reflection feature that shows up in soundings of shallow sub-bottom records across many continental margins. At Blake ridge¹, drilling into a bottom-simulating reflector² (BSR) has fuelled a long-standing discussion (or argument) about how much methane a gas-hydrate layer can hold. Geophysical results lower previous estimates by a factor of three³. But now Dickens and colleagues (on page 426 of this issue⁴) bump estimates back up again with samples of the material recovered in a pressurized core barrel.

Gas hydrate forms under conditions of high pressure and low temperature, when a regular lattice of water molecules includes a molecule of gas — often methane, but also higher hydrocarbons up to pentane⁵. The resulting solid is stable at temperatures below about 7 °C and pressures greater than 50 atmospheres. In marine sediments, the depth below the ocean bottom at which hydrates can form depends on the gas concentration, geothermal gradient and ambient pressure. Below this depth, methane exists as free gas, above as hydrate. The contrast between sound velocity in sediment and in a hydrate/free-gas layer generates a distinctive seismic reflector — the phase-boundary conditions tend to track a constant depth beneath the water bottom in a given region, often cutting across tilted bedding planes, faults, and other reflectors. Hence the term bottom-simulating reflector.

These characteristics were apparent in the first geophysical detection of the 'type specimen' BSR on the Blake ridge. Early deep-sea drilling provided chemical evidence that gas hydrate could account for

the anomalies. Similar BSRs appear in many other locations⁶, and conditions that should allow hydrate formation prevail across most of the ocean below 500 m, so the potential gas-hydrate province is vast: most estimates of the total carbon stored^{7–9} range from 1,700 to 11,000 billion tonnes — and there is one estimate of 4,100,000 billion tonnes.

The difficulty is in determining how much methane, free or in hydrates, will produce the detected change in sound velocity. Gas hydrate and the associated free gas want to destabilize — sometimes vigorously — when removed from their stratigraphic bed and brought to the surface. Trying to calculate original concentrations from the remaining soup is not easy. Furthermore, sedimentary anomalies that have nothing to do with hydrate can produce BSR-like seismic signatures. Direct evidence is clearly needed.

A BSR is a hydrocarbon reservoir that creates its own 'seal' when the hydrate layer traps free gas below. Typically, traps fill when gas or oil migrate upward along fault planes. But if a typical BSR cuts across many faults, what source fills the self-made reservoir¹⁰? It turns out that most BSRs are higher up than the theoretical depth of the phase boundary — in other words, most hydrate could have formed deeper than where it is actually found. So either some BSRs have migrated well above the point where hydrate formation ought to have stopped further permeation, or they are relicts of an earlier, different temperature and pressure regime — perhaps dating from the Pleistocene. If they are relicts, what potential is there for this sleeping giant reservoir of greenhouse gas to awaken?

To address these and other questions, in 1995, Ocean Drilling Program Leg 164 drilled a series of holes through the Blake ridge hydrate layer. Holbrook and collaborators³ measured vertical seismic velocities in these sections, and concluded that hydrate fills

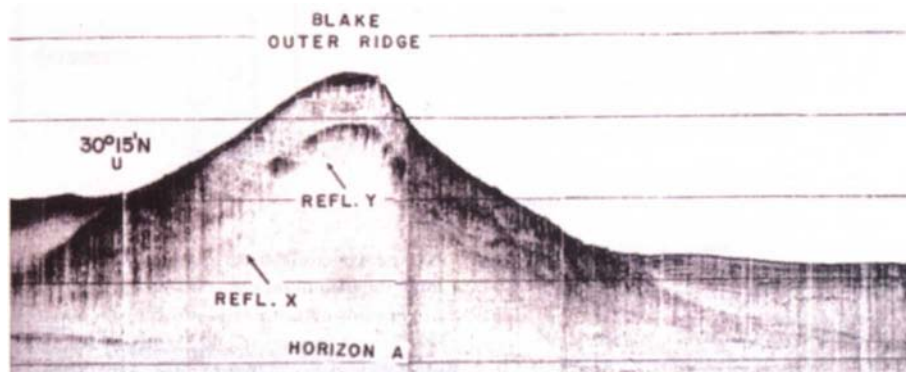


Figure 1 Seismic-reflection profile of Blake ridge in the Atlantic Ocean off the southeastern United States, circa 1970. The phase boundaries of a layer of gas hydrate (reflection Y) and an even thicker layer of free gas (reflection X) seem to simulate the ocean bottom. (From ref. 2.)

2–7% of the pore volume in the hydrate layer and gas bubbles fill 1% of the free-gas zone beneath. Multiplying through by layer thicknesses and concentrations, they speculate that the ~100,000 km² of the southeastern US margin believed to contain hydrate may hold 40 billion tonnes of methane carbon.

Dickens *et al.* used a pressurized coring device to recover samples of the BSR *in situ* pressure, and so were able to measure the quantity and composition of gas directly. They concluded that hydrate occupies 0–9% and gas occupies 12% of pore volume in the two zones. Extrapolating from somewhat different assumptions, they arrive at 35 billion tonnes of carbon in roughly a quarter of the total area. So the best direct evidence from the original BSR confirms that methane volumes can be very large indeed. Generalizing from these cores to the entire Blake ridge — and the planet — will no doubt occupy future studies.

Judging by newly scheduled conferences and publications, there is an awakening of interest in oceanic gas hydrate, scientific and industrial. When we contemplate layers of hydrocarbon spanning millions of square kilometres, the inclination to dream about how many cities might be lighted with the stuff can prove irresistible. We should resist, however, because submarine gas hydrate is a very special sort of hydrocarbon deposit. It is thinly spread, found at great depth in the ocean, and is dangerous if drilled. Attempts to exploit terrestrial hydrate deposits in Siberia have produced decidedly mixed results.

Deposits like the Blake ridge BSR do not resemble economic hydrocarbon reserves as we presently know them. Their practical importance remains questionable, but most immediately it lies in understanding hydrate formation — to stop hydrates from plugging up deep-water pipelines, for example, or even to induce hydrate formation for transport purposes, as an alternative to liquid natural gas — and above all in unravelling the origins and probable fate of this enormous component of the carbon cycle. □

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Membrane proteins

Structure of a molecular hole-punch

Mark S. P. Sansom

Several bacterial toxins work by punching a hole in the membrane of their target cell. This produces a protein-lined ion channel (or pore), which dissipates the electrochemical gradients across the membrane and results in the death of the cell under attack. To do the job, a toxin has to reach its target membrane and then insert itself into the membrane so as to form a channel. For those toxins such as the colicins, whose target cells are Gram-negative bacteria, there is another complication in that the toxin has to bind specifically to the outer membrane of the cell, but then has to form an ion channel in the inner membrane.

Wiener *et al.* (page 461 of this issue¹) have solved the crystal structure of an intact colicin molecule, that of colicin Ia, and the structure suggests an ingenious way in which these tasks are performed. It reveals the three domains responsible for the three stages of attack on the target membrane: binding of colicin Ia to its outer-membrane receptor; translocation of the toxin's channel-forming domain from the outer to the inner membrane; and channel formation in the inner membrane. As well as these three distinct domains, colicin Ia has a pair of exceptionally long α -helices that enable the protein to span the gap between the outer and inner membranes.

The carboxy-terminal domain of a number of different colicins (A, E1, Ia) forms ion channels in lipid bilayers², and previous crystallographic³, spectroscopic⁴ and electrophysiological⁵ studies had concentrated on the structure of fragments of this domain and the mechanism of channel formation. Consequently, these studies neglected the rather more complex question of how the channel-forming domain is delivered to the inner membrane. The structure described by Wiener *et al.* is a major advance because we now see the molecular make-up of all three domains — the amino-terminal translocation (T) domain (residues 1–225); the central, receptor-binding (R) domain (residues 282–385); and the carboxy-terminal, channel-forming (C) domain (residues 450–626).

Both the T and C domains are largely α -helical, whereas the R domain contains an amphipathic β -hairpin. The T and R domains are linked by a long (about 160-Å) α -helix, which coils around a similar helix connecting the R and C domains. Thus, the C and T domains pack side-by-side, at least in the crystal structure, and are about 160 Å from the R domain, which lies at the other end of the molecule. Significantly, the distance separating the outer from the inner membrane is thought to be about 150 Å. So the separation of the R and C domains in the

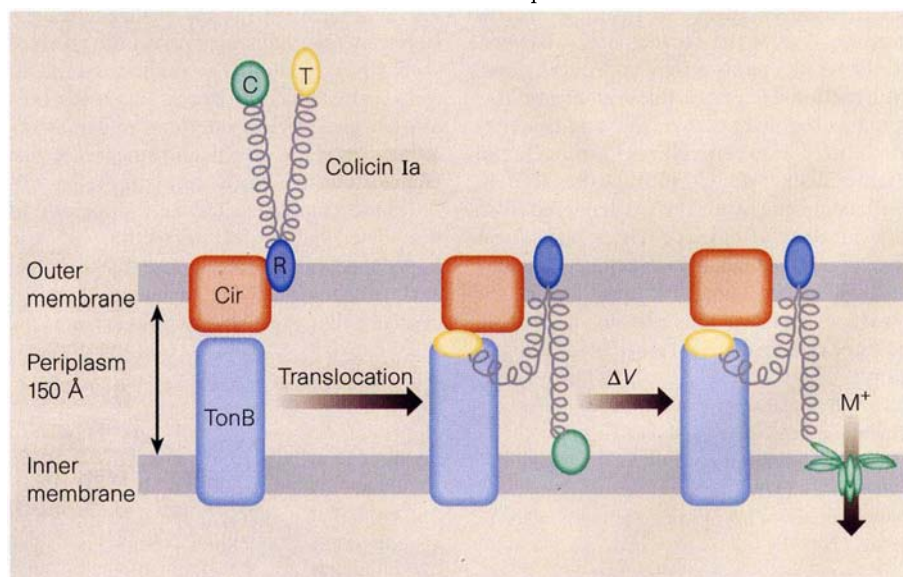


Figure 1 A bacterial toxin in action — scheme for the interaction of colicin Ia with the outer and inner cell membranes of a target bacterium. The receptor-binding (R) domain binds to its receptor, Cir. Binding of the translocation (T) domain to the TonB protein is followed by translocation of the channel-forming (C) domain to the inner membrane. Interaction of the C domain with the inner membrane, in the presence of a transmembrane voltage difference, ΔV , results in repacking of the constituent helices of the C domain to form a transbilayer pore, through which ions (M^+) can flow. This leads to dissipation of electrochemical gradients across the inner membrane and death of the cell.

intact protein matches the separation of their target outer and inner membranes respectively. By combining the crystallographic structure with the results of other, less direct experiments, and taking this line of thinking further, a picture of the events underlying the action of colicin Ia emerges (Fig. 1).

The colicins exploit the machinery of the target cell to get across the periplasmic space separating the outer and inner membranes. The first event is binding of the toxin's R domain to its receptor, an outer-membrane protein called Cir which forms part of a metabolite transport system. In the absence of colicin Ia, Cir binds to a second protein, TonB, by a five-residue motif in the Cir sequence known as the TonB box. TonB is an inner-membrane protein but spans the periplasmic space and couples the electrochemical gradient across the inner membrane to transport through Cir. After colicin Ia has bound to Cir by its R domain, a TonB box within the T domain displaces the TonB box of Cir and binds to TonB.

So the T domain allows colicin Ia to exploit the TonB system and translocate part of the toxin across the periplasm, while the R domain remains bound to Cir. This is probably achieved using the two 160-Å-long helices that link the R domain with the other two domains of colicin Ia, thus allowing the protein to span the gap. What is less clear is whether both the T domain and the C domain are translocated, or whether (as shown in Fig. 1) the T domain remains bound to TonB.

Having reached the inner membrane, the C domain undergoes a series of structural rearrangements which have been probed by analysing C-domain fragments of various colicins^{2,6}. The carboxy-terminal part of colicin Ia exhibits the same fold (a ten-helix bundle) as the corresponding domains of colicins A (ref. 3) and E1 (ref. 7). These helices repack upon interacting with the inner membrane. A central helix hairpin within the ten-helix bundle (C8–C9 in Wiener and colleagues' nomenclature) is highly hydrophobic and spontaneously inserts into lipid bilayers. This is followed by voltage-driven insertion of further helices. In particular, a second hairpin, C6–C7, inserts and is thought to associate with C8–C9 to form a transmembrane four-helix bundle. This may act as an ion channel, allowing dissipation of electrochemical gradients across the inner membrane. Thus, colicin Ia exploits the TonB system to translocate the C domain across the periplasm, and it exploits the voltage difference across the inner membrane to drive structural rearrangements of the C domain that result in channel formation.

The structure of intact colicin Ia shows that the function of this protein is more than

the sum of its parts—a salutary lesson, given the increasing publication of structures of isolated domains of proteins. However, the new work also nicely illustrates the point that a crystal structure often forms the beginning rather than the end of a story.

In this case, two important questions remain unanswered. How are the long inter-domain helices used to exploit the TonB translocation system? And what are the molecular events that lead to the formation of an ion channel (whose structure remains unknown) once the C domain has reached the inner membrane? This second process involves the dynamic interactions of helices and a bilayer in response to a transmembrane voltage. As this is an inherently non-equilibrium system, it is difficult to see how crystallographic studies will answer the question, although simulation studies of helix–bilayer–voltage systems may tell us more about such events⁸. What

is unequivocally clear, however, is that the colicins will remain an ideal test system for probing the protein interactions within membranes that occur in such dynamic processes as translocation and insertion. □

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Nerve regeneration

Help from within for damaged axons

Yves-Alain Barde

Continuity is essential for signals to be conducted along axons. If a vertebrate axon is cut, the segment beyond the cut degenerates, and much work has concentrated on improving conditions for the re-growth of axons by modifying their environment. But new *in vivo* studies, including one by Chen *et al.* described on page 434 of this issue¹, reveal that molecules intrinsic to the damaged neurons also affect their regenerative properties.

In birds and mammals, neurons of the peripheral nervous system (PNS) grow new axons after injury, and re-establish functional connections. But not so in the central nervous system (CNS), where the result is persistent loss of function—the consequences of an injury to the spinal cord, which harbours countless bundles of axons, are only too familiar. So do neurons in the PNS and CNS differ fundamentally in their ability to generate new axons after lesion?

Clearly not. First, although the cell bodies of many sensory neurons lie in the PNS, their axons divide into one branch that remains in the PNS and one that projects into the CNS. After section, only the PNS branch is able to regenerate². Second, grafting experiments show that CNS neurons, when provided with the type of environment normally encountered by PNS neurons, will regenerate an axon³. Both observations suggest that the CNS environment somehow doesn't allow axons to regrow. Axons of both the PNS and CNS are covered with a myelin sheath, which however is generated by Schwann cells in the PNS and oligodendrocytes in the CNS, and

CNS myelin has been identified as one of the factors responsible for the failure of CNS neurons to regenerate⁴. Indeed, blocking inhibitory components of CNS myelin with the appropriate antibodies allows long-distance growth of at least some axons in a cut spinal cord⁵.

But although the importance of extrinsic determinants in axonal elongation is not questioned, it has become clear that it is worth taking a close look at molecules intrinsic to damaged CNS neurons. In particular, Aigner *et al.*⁶ demonstrated that overexpression of the protein GAP-43 (for 'growth-associated protein') in transgenic mice causes sprouting of CNS axons beyond the borders of their normal territory. What is more, substantial axon regrowth was observed in these mice when the axons of sensory neurons projecting into the CNS were cut.

Chen *et al.*¹ now report on experiments using the retina and the *bcl-2* gene. When young embryonic explants of the retina are co-cultured with the superior colliculus (one of the targets in the brain normally innervated by retinal ganglion cells), axonal invasion of the target takes place. But this doesn't happen when explants from mice carrying a deletion in the *bcl-2* gene are used. The retinal ganglion cells express high levels of Bcl-2 protein, but only early in development, and longer-lasting overexpression of the *bcl-2* gene leads to target innervation at a stage when ganglion cells from control mice have long ceased to do so.

Finally (and most importantly), after section of the axons of retinal ganglion cells

in vivo, re-innervation of the superior colliculus takes place in mice overexpressing Bcl-2. Although the distances involved might seem modest (half a millimetre or so), and the experiments have been performed in very young animals, the results are striking when one considers that, in controls, all axons stop sharply at the lesion.

But what is *bcl-2*? This gene was discovered as a result of a chromosomal translocation that placed it under the inappropriate control of an immunoglobulin gene promoter, leading to B-cell lymphoma (hence its name)⁷. Using biochemical pathways that remain largely unclear, Bcl-2 can block programmed cell death, not only of B cells, but also of many other cell types including neurons. Significantly, *bcl-2* is related in sequence to the *Caenorhabditis elegans* gene *ced-9*, which is needed to prevent programmed cell death in nematodes⁸. However, the effects of Bcl-2 on axonal elongation reported by Chen *et al.* do not seem to be a consequence of inhibition of programmed cell death. In particular, when the death of cultured retinal ganglion cells is prevented by a protease inhibitor, axonal elongation does not occur. Also, there is no loss of retinal ganglion cells in *bcl-2* null mutants during the developmental period studied⁹.

Clearly, Bcl-2 involvement in axonal elongation will need to be tested in other circumstances, in particular in older animals and in experiments in which axons are cut closer to the cell body. Also, it is now possible to culture what are essentially pure postnatal, rodent retinal ganglion cells for extended periods¹⁰. Culturing such cells from animals that are both deficient in and overexpress *bcl-2*, and comparing axonal elongation in the two, would also help to establish the degree to which *bcl-2*-dependent outgrowth is cell autonomous.

When considering the properties of Bcl-2 protein, it is worth remembering that extracellular signals have long been known to be involved in both neuronal survival and neurite elongation. For example, nerve growth factor can both prevent the death of PC12 cells and promote neurite outgrowth^{11,12}. Also, brain-derived neurotrophic factor can promote neurite outgrowth of post-mitotic sensory neurons, as well as block programmed cell death¹³. Both actions can be mimicked by an oncogenic form of the Ras protein introduced into sensory neurons¹⁴, and recent data indicate that Bcl-2 associates with BAG-1 protein, which, like Ras, is known to activate the kinase Raf-1¹⁵.

All in all, finding ways to upregulate effective levels of Bcl-2 in neurons might be useful not only with a view to preventing neuronal cell death, but also to favouring axonal elongation in the CNS. When this approach is

combined with strategies for modifying the environment of injured neurons, we can expect progress towards the goal of efficiently restoring axonal elongation in the damaged CNS. There are lessons to be learned from other sources as well. Those working with amphibia and fish have long told us that functional restoration works well in the adult CNS; moreover, we should not forget that massive axonal elongation occurs in the CNS of every species during development. □

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Palaeoclimatology

Near to the edge of an ice sheet

Steven W. Hostetler

During the last deglaciation (from about 16,000 to 6,000 radiocarbon years before present, ¹⁴C yr BP) the Laurentide ice sheet in northern North America existed at a time when July solar-radiation levels were higher than at present. The result was unusual climatic conditions that changed markedly over relatively short distances. On page 423 of this issue¹, Levesque *et al.* report evidence of remarkably large differences in July temperatures within a transect of five closely spaced lakes situated near the ice sheet in Maritime eastern North America (see map on page 424). Their findings document a particularly steep, late-glacial climatic gradient, and provide a challenge for understanding and modelling the high-resolution aspects of palaeoclimate near the ice sheet.

Pollen reconstructions² and simulations with atmospheric general circulation models³ (AGCMs) for the period of deglaciation yield temperature gradients of about 0.6 °C per 100 km for eastern North America at 12,000 ¹⁴C yr BP. Closer to the Laurentide ice sheet, model results indicate a gradient of 2 °C per 100 km at the same time; for the mid-continent at 14,000 ¹⁴C yr BP, a gradient of about 1.5 °C in 100 km has been reconstructed from fossil beetle assemblages⁴.

Based on their analyses of aquatic midge larvae in cores from the five lakes, however, Levesque *et al.* come up with July lake temperature differences of 11 °C in 240 km and 9 °C in 55 km, gradients of 4.6 °C and 16 °C per 100 km, respectively. For perspective, today in eastern North America, temperature differences of this magnitude occur over about 15° in latitude, a distance of more than 1,500 km.

The findings of Levesque *et al.* point to the interplay of different scales of palaeo-

climate over a period of several thousand years. The temperature gradients between the lakes and the general, correlative patterns of lake cooling through the Killarney Oscillation⁵ (11,400 ¹⁴C yr BP) and Younger Dryas (11,000–10,000 ¹⁴C yr BP) are evidence of the shifting dominance between regional-scale (mesoscale) and large-scale (synoptic) atmospheric circulation. From 12,000 to 9,000 ¹⁴C yr BP, solar-radiation levels increased in the Northern Hemisphere as a result of changes in Earth–Sun geometry. Compared with the present, average solar-radiation levels in July were 8 per cent higher while average winter levels were 8 per cent lower⁶. Higher summer insolation increased continental heating away from the ice sheet. AGCM simulations³ for 12,000 ¹⁴C yr BP indicate that, during July, south-to-south-westerly winds at the surface were focused on the region of the lakes; the strength of this flow is reduced in simulations for 9,000 ¹⁴C yr BP. In addition, this region is adjacent to the area where oceanic circulation gives rise to the steepest spatial gradients and greatest annual range of sea-surface temperatures in the North Atlantic^{7,8}.

What were the consequences for the climate at the edge of the ice sheet? Warm continental and marine air would have been carried into and over the colder, denser air along the ice sheet by the synoptic-scale wind flow at 12,000 ¹⁴C yr BP, resulting in a stationary mesoscale front having characteristics similar to a mid-latitude warm front (Fig. 1). Warm fronts are typically associated with light precipitation or drizzle, so it is likely that the main effect of this front would have been to form ground fog and low stratus clouds. Near the ice sheet, widespread clouds would have reflected solar radiation and thereby reduced the amount of radiation

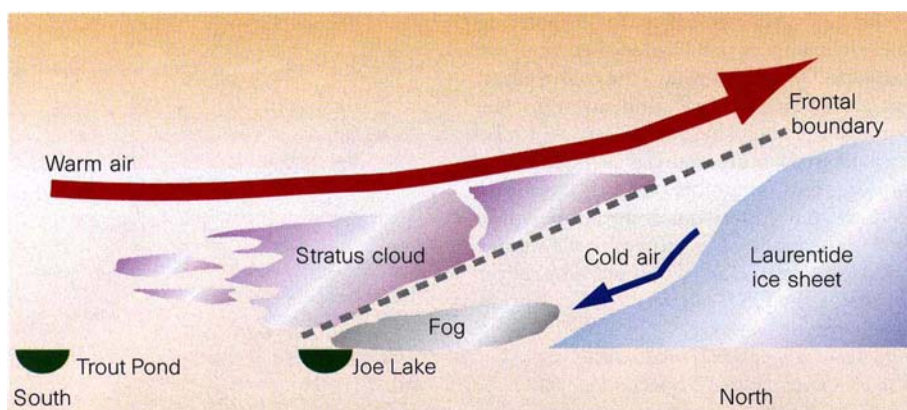


Figure 1 Idealized stationary warm front along the Laurentide ice sheet at 12,000 ^{14}C BP, as surmised from the data of Levesque *et al.*¹ discussed here. The features of the front are patterned after those of the classical Norwegian model of mid-latitude warm fronts¹¹.

available for lake heating. Fog along the ice sheet and in the Gulf of St Lawrence, caused by differences between air and sea-surface temperatures, may have reduced radiation even further. As a result of limited radiation and cold temperatures near the ice sheet, the northernmost lake (Joe Lake) remained about 10 °C colder than present throughout the period. The temperatures of the lakes to the south indicate that the front dissipated and cold temperatures, low clouds and fog gave way to warm and relatively clear conditions. Increased solar radiation away from the front heated the southernmost lake (Trout Pond) to temperatures essentially the same as today.

As is evidenced by higher levels of the lakes in the transect^{1,5}, regional net moisture (precipitation minus evaporation, $P-E$) was greater than present during the late glacial². Closer to the ice sheet, greater $P-E$ values reflect both greater precipitation and, because lake temperatures were colder, lower evaporation. To the south, however, July evaporation rates of Trout Pond should have been nearly the same as at present, which suggests that $P-E$ was enhanced by increased precipitation that probably fell during the winter and spring.

During the Killarney Oscillation^{1,5} and Younger Dryas⁹, stronger and colder synoptic-scale circulations associated with cooling of the North Atlantic replaced the mesoscale pattern. In the Younger Dryas, the lakes cooled by as much as 15 °C and the colder temperatures suggest the lakes may have been covered with ice for much of the year. The lakes cooled to a lesser degree in the Killarney Oscillation, and it is during this time that the steepest gradient (16 °C in 100 km) existed. How such a gradient was maintained is puzzling if, as hypothesized^{1,5}, the Killarney Oscillation affected the entire North Atlantic region in the same way as the Younger Dryas; the gradient may, however, have been associated with changes in oceanic circulation. The mesoscale pattern was re-established between the

Killarney Oscillation and Younger Dryas, and thereafter was replaced by conditions similar to those at present as the ice sheet melted.

Results such as those of Levesque *et al.* will stimulate the continued refinement of climatic hypotheses, and they highlight the need to improve Earth-system models in three areas. First, the grid spacing of a typical AGCM (400 to 700 km) is far greater than the 240-km length of the lake transect, which makes interpretation of model results at this scale uncertain. Second, clouds and fog probably played an important role in the climatology recorded by the lakes, but it remains difficult to incorporate the physics concerned into climate models. Last, oceans are a dominant part of the climate system, so it is necessary to couple

atmospheric and oceanic models to achieve accurate simulations of climate.

Progression to higher-resolution regional¹⁰ and global atmospheric models, and the application of coupled atmosphere-ocean models in palaeoclimate experiments, are being facilitated by rapidly increasing computer capacities, and improving cloud processes in climate models remains a very active area of atmospheric research. Meeting these modelling challenges will contribute substantially to our confidence when looking into the future, especially if the models are able to simulate past climates for which there are no modern analogues. □

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Calcium channels

Integration hot-spot gets hotter

Kathleen Dunlap

Almost exactly eleven years ago, voltage-gated Ca^{2+} channels were first reported to be modulated by guanine-nucleotide-binding (G) proteins¹. Such modulation by G-protein-coupled receptors has proven to be as fundamental and widespread as the intrinsic voltage-dependent regulation of Ca^{2+} channels. A variety of signalling pathways that affect Ca^{2+} channels have been described — some that enhance and others that inhibit, Ca^{2+} influx. The diversity in this array of modulatory pathways is a reflection of the diverse physiological functions triggered by Ca^{2+} , from exocytosis, to contractility, to gene transcription. Researchers have now begun to tease apart this web of interconnected signalling pathways in search of molecular explanations for the different forms of channel regulation.

On pages 442 and 446 of this issue, Zamponi *et al.*² and De Waard *et al.*³ explain

how one form of G-protein-mediated inhibition that is common among neuronal Ca^{2+} channels may occur. This inhibitory pathway is activated by a variety of neurotransmitter receptors through G_o -type G proteins^{4,5}. Inhibition is thought to occur through a direct interaction between the Ca^{2+} channel complex and the $\beta\gamma$ subunits that are released from activated G-protein heterotrimers ($\text{G}\alpha\beta\gamma$)^{6,7}, and the new data provide the first direct confirmation of this hypothesis.

Both groups^{2,3} studied this signalling pathway by expressing components of G proteins and Ca^{2+} channels in transfected cells and then monitoring the interactions between them using a combination of biochemical and electrophysiological methods. They analysed the binding of radiolabelled G-protein subunits to various Ca^{2+} -channel α_1 subunits that were expressed as glutathione *S*-transferase (GST) fusion proteins, and found that $\text{G}\beta$ and $\text{G}\beta\gamma$ (but not

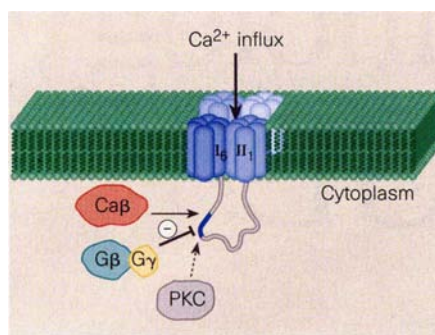


Figure 1 Several classes of Ca^{2+} channel exist (A, B, C, D, E and S). They all contain a transmembrane α_1 subunit that is made up of four homologous repeats (I–IV), each of which comprises six putative membrane-spanning helices. The intracellular cytoplasmic 'linker' domain is a stretch of >100 amino acids that links helix 6 of repeat I (I_6) to helix 1 of repeat II (II_1). The highlighted region of this I–II linker is a hot-spot for molecular integration — it binds Ca^{2+} -channel β subunits (Ca β) which enhance channel function. Both Zamponi *et al.*² and De Waard *et al.*³ have shown that in class A, B and E channels it also binds G-protein $\beta\gamma$ -subunit complexes (G $\beta\gamma$), which inhibit channel function. Additionally, phosphorylation of this domain by protein kinase C (PKC) eliminates G $\beta\gamma$ -mediated inhibition of the Ca^{2+} current.

G α or G γ alone) bind to the cytoplasmic linker region that is found between transmembrane repeats I and II (Fig. 1) of some Ca^{2+} -channel classes (α_{1A} , α_{1B} and α_{1E}) but not of others (α_{1C} , α_{1D} and α_{1S}).

De Waard *et al.*³ noted that the amino-terminal region of the I–II linker contains a sequence that includes the QXXER sequence, which is essential for G $\beta\gamma$ to bind to adenyl cyclase⁸. By mutating Q and R in this domain, along with several flanking amino acids, they were able to prevent the binding of G $\beta\gamma$ to this region of the I–II linker protein derived from the α_{1A} Ca^{2+} channel. Interestingly, a second (carboxy-terminal) region of the α_{1A} I–II linker also binds G $\beta\gamma$, even when the amino terminus is mutated.

Electrophysiological recordings allowed the two groups to move from the realm of protein-binding assays to that of physiological function. Zamponi *et al.*² showed that when peptides containing the QXXER sequence are injected into cells, they interfere with G $\beta\gamma$ -mediated inhibition of α_{1A} and α_{1B} Ca^{2+} currents. Taking this a step further, De Waard *et al.*³ found that the point mutation in α_{1A} that eliminates G $\beta\gamma$ binding to the amino terminus of the I–II linker also prevents the G-protein-dependent inhibition of α_{1A} currents that occurs when G proteins are directly activated by GTP γ S. So the direct binding of G $\beta\gamma$ to the Ca^{2+} -channel α_1 subunit seems to promote a voltage-dependent inhibition of current that is conserved among class A, B and E channels,

but not among class C, D or S channels.

The I–II linker domain has other functions within Ca^{2+} channels. The Ca^{2+} -channel β subunit interacts with the α_1 subunit I–II linker and enhances the Ca^{2+} current⁹ (Fig. 1). Together with these data, the new findings^{2,3} provide a structural explanation for the observation that the β subunit interferes with G-protein-mediated inhibition of Ca^{2+} -channel function¹⁰. Zamponi *et al.* show that some of the I–II linker peptides that interrupt channel modulation by G proteins are also substrates for protein kinase C. Phosphorylation of these peptides *in vitro* reduces their ability to inhibit G-protein-dependent modulation. This explains why, in some cells, activation of protein kinase C can prevent the voltage-dependent inhibition of Ca^{2+} currents that is produced by transmitters¹¹.

Some of the new results provide additional food for thought. First, the function of the carboxy-terminal binding site in the I–II linker is not known. De Waard *et al.*³ report that the binding of G $\beta\gamma$ to this site is unaffected by a mutation elsewhere which eliminates G-protein-dependent inhibition. Paradoxically, Zamponi *et al.*² find that G-protein-mediated inhibition is blocked by a peptide from this carboxy-terminal I–II linker. Moreover, a second peptide that is derived from an amino-terminal domain which — according to De Waard *et al.* — does not bind G $\beta\gamma$, also interrupts G-protein-mediated inhibition. The mechanisms of peptide action, and whether these sequences in the intact channel protein bind G $\beta\gamma$ and/or regulate function, are unknown.

Second, transmitter-mediated inhibition seems to be more effective than that produced by the direct application of purified G $\beta\gamma$ to Ca^{2+} channels. Furthermore, even at very high concentrations of G $\beta\gamma$ ^{6,7,12}, α_{1B} currents are modulated more effectively than are α_{1A} or α_{1E} currents^{2,3}. Finally, the function of the Ca^{2+} -channel β subunit and its potential role in influencing the relative sensitivity of α_1 subunits to modulation by G $\beta\gamma$ are unknown.

We have much to learn, as evidenced by a recent paper from Tsien and co-workers¹² whose results seem to contradict those reported here. Tsien's group expressed Ca^{2+} -channel-protein chimaeras in *Xenopus* oocytes to investigate the domains necessary for G-protein-mediated inhibition. They found that replacing the I–II linker from α_{1B} with that from α_{1C} — a channel not inhibited by G proteins — had no effect on G-protein-mediated inhibition of α_{1B} currents. To eliminate G-protein-mediated inhibition, they had to replace both the I–II linker and a second domain in the α_{1B} carboxy terminus with the equivalent domains from α_{1C} . So, in their studies, many sites were essential for G-protein-dependent inhibition.

It seems unlikely that this discrepancy



100 YEARS AGO

Mr. J. L. Williams has a very curious note in the number of the *Journal of Botany* for January, on the drunken habits of certain humble-bees. The intoxicant is the honey produced by the crowded flowers of the capitulate heads of certain Compositae (*Carduus nutans* and *lanceolatus*, and *Centaurea scabiosa*), and Dipsacaceae (*Scabiosa succisa*). The intoxication is indicated by rolling on the back, striking the legs wildly in the air, and general helplessness. The bees rapidly recovered from the effects, and, in most cases, were eager to repeat the debauch; but one individual, which had been shut up in a vasculum with copious supplies of *Centaurea scabiosa*, manifested, the next morning, a praiseworthy remorse and disgust, "raising its head and fore-legs as high as it could above the plants, then precipitately hurrying away as soon as released." The most dissolute species appears to be the neuter of *Bombus lapidarius*. The author suggests that this may in time become a normal mode of cross-pollinating the flowers in question. From *Nature* 28 January 1897.

50 YEARS AGO

Dr. A. Parker, director of fuel research, Department of Scientific and Industrial Research, speaking on "Oil from Coal in Germany" at the Fuel Luncheon Club on January 23, stated that Germany developed before the War two synthetic processes, hydrogenation and the Fischer-Tropsch process for the production of petroleum from coal, and many large plants were constructed. At the time of maximum production during the early months of 1944, these two processes together provided oil at a rate of nearly four million tons a year.... Dr. Parker stated that bombing attacks between May and September 1944 caused a reduction in German production of synthetic oil from a rate of nearly four million tons a year to only about 300,000 tons. During the following months, with a reduction in bombing and determined efforts in Germany to repair damage, production again rose to a rate of one million tons a year in November 1944, after which it again fell to 150,000 tons a year at the end of February 1945. It is certain that this systematic destruction of German oil plants was one of the main factors in hastening the defeat of Germany. From *Nature* 1 February 1947.

stems from the relatively minor differences between the α subunits studied in the different laboratories, although discrepancies could perhaps result from variations in the relative expression levels of the transfected signalling molecules. For example, the I-II linker might simply be a high-affinity anchoring site for G β γ , so that the G-protein subunits can be presented to a lower-affinity modulatory site that is not detected by the binding assays reported here. If so, the anchoring site may not be needed when there are sufficiently high concentrations of G β γ — such as those achieved by Tsien's group through over-expression methods.

As modulation of Ca²⁺ channels probably depends on the integration of many low-affinity molecular interactions, the functional phenotype of a channel will vary because of differences in the relative concentrations of these signalling proteins. So in future studies it will be necessary to quantify the levels of expressed proteins and, when possible, to titrate them into the ranges

typically found *in vivo*. Combining information from investigations of transfected cells such as those reported here, with studies of primary cells (where protein expression levels are by definition 'normal'), should help to unravel the complex molecular interactions that alter Ca²⁺-channel function, and this may allow us to define the physiological rationale for their existence. □

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Plant disease resistance

A kinase with keen eyes

Jonathan D. G. Jones

It is always satisfying when the molecular interactions that explain a genetic phenomenon of fundamental and applied significance are revealed. Four recent papers^{1–4} so illuminate the study of disease resistance in plants, showing that pathogen-encoded proteins are introduced into plant cells where they can be recognized by plant-encoded receptors to initiate a defence response.

Many plants contain disease-resistance (R) genes that confer resistance to fungal, bacterial, viral or invertebrate pathogens. In the 1940s H. H. Flor showed that for the interaction between flax and the fungal pathogen flax rust, not only are the R genes dominant, but mutations that enable the rust to overcome an R gene are recessive. This led to the current view that pathogens contain a variety of molecules — specified by dominant *avirulence* (*avr*) genes — that contribute to pathogenicity. A particular *avr* gene product will trigger a defence response if the plant has evolved a corresponding R gene that recognizes it, and this type of interaction is defined as 'gene-for-gene' resistance. It was anticipated that the R-gene products would be receptors for ligands specified by the *avr* genes⁵, and that ligand binding would stimulate a signalling cascade leading to the activation of defence mechanisms that may result in the death of infected cells. R-gene-dependent defence mechanisms are often associated with a hypersensitive response (HR) or local

cell death at sites of attempted pathogen ingress.

A number of R genes have been isolated^{6,7}. Many of them encode transmembrane or cytoplasmic proteins containing leucine-rich repeats (LRRs) which could participate in the protein-protein interactions that might underlie gene-for-gene recognition. For example, the product of the tomato *Cf-9* gene⁸ for leaf-mould resistance is predicted to be a transmembrane protein with extracellular LRRs. The probable ligand is the 28-amino-acid secreted peptide product of the fungal *avr9* gene⁹. The ligand for the predict-

ed cytoplasmic product of the tobacco N gene for resistance against tobacco mosaic virus¹⁰ is likely to be derived from the viral replicase¹¹ (Table 1).

The first 'gene-for-gene' R gene to be cloned was the tomato *Pto* gene¹², which confers resistance to strains of the bacterial pathogen *Pseudomonas syringae* pathovar *tomato* that carry the *avrPto* gene. Unlike either of the LRR classes of R genes (Table 1), *Pto* is a member of the Raf family of serine-threonine protein kinases. The *Pto* protein lacks an apparent regulatory domain, which is surprising if the anticipated functions of recognition and signal transduction are located in distinct regions. *Pto* function requires *Prf*¹³, a closely linked gene that encodes a protein with homology to R genes of the cytoplasmic LRR family^{6,7}. Also, two-hybrid analysis in yeast has shown that *Pto* interacts with — and phosphorylates — *Pti1*, the overexpression of which also confers (or enhances) resistance to strains containing *avrPto*¹⁴.

*AvrPto*¹⁵ is one of a number of bacterial *avr* gene products that have long provided a puzzle because they seem to be cytoplasmic proteins that lack a signal peptide, and they have no apparent means by which to present themselves to any recognition apparatus in the plant cell. For the *avr* gene products to be recognized by the plant, the bacterium must also carry functional *hrp* genes, so called because they are required both to elicit an HR and for pathogenicity¹⁶. The *hrp* genes show sequence homology to certain genes encoded by mammalian-cell pathogens (such as *Shigella* and *Yersinia*), that encode a type-III secretion system to deliver bacterial virulence factors into the eukaryotic cytoplasm¹⁷ (Fig. 1, overleaf).

Could this homology mean that bacterial plant pathogens have a similar apparatus, to pump 'pathogenicity' proteins into plant cells — proteins that the plant could evolve the capacity to recognize? And could gene-

Table 1 Plant disease resistance genes and the corresponding *avr* gene products

Plant	R gene	Structure	Pathogen	<i>avr</i> gene
Tomato	<i>Cf-9</i>	Transmembrane protein with extracytoplasmic LRRs	Leaf mould	<i>avr9</i>
Tobacco	<i>N</i>	Cytoplasmic NBS*/LRR	Tobacco mosaic virus	Viral replicase?
Tomato	<i>Pto</i>	Serine-threonine protein kinase	<i>P. syringae</i>	<i>avrPto</i>
Tomato	<i>Fen</i>	Serine-threonine protein kinase	Unknown, but confers sensitivity to Fenthion insecticide	
Pepper	<i>Bs3</i>	?	<i>Xanthomonas</i>	<i>avrBs3</i>
<i>A. thaliana</i>	<i>Rps2</i>	NBS*/LRR	<i>P. syringae</i>	<i>avrRpt2</i>
<i>A. thaliana</i>	<i>Rpm1</i>	NBS*/LRR	<i>P. syringae</i>	<i>avrB</i> <i>avrRpm1</i>

* Three of the four classes of resistance genes encode LRRs (refs 6 and 7). One class also carries a nucleotide binding site (NBS), and is believed to be cytoplasmic.

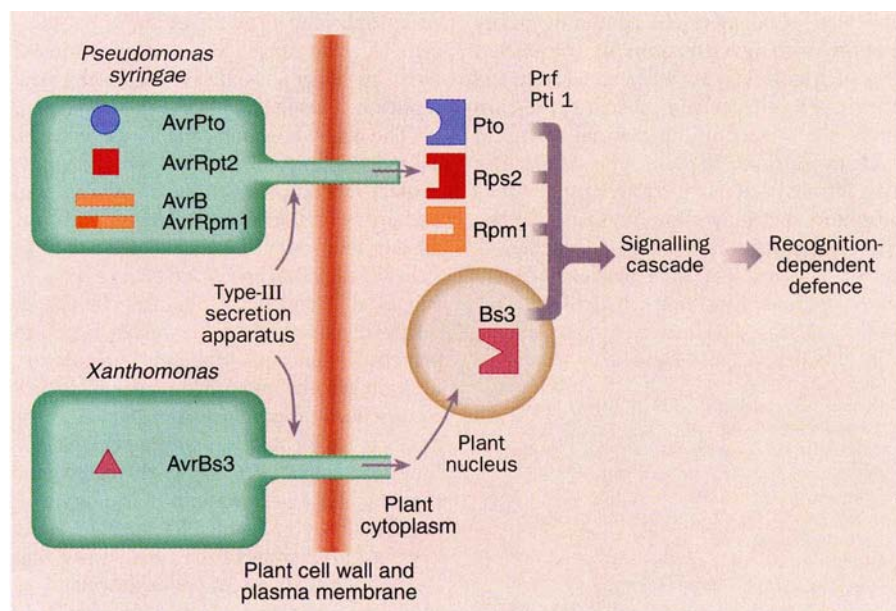


Figure 1 The current view of the recognition events that enable plants to detect and respond to pathogenic bacteria. The *avrPto*, *avrRpt2*, *avrB* or *avrRpm1* genes (from *Pseudomonas*) or *avrBs3* (from *Xanthomonas*) are expressed in the bacterial cell and transferred into the plant cell by a type-III secretion mechanism resembling that used by the mammalian pathogens *Yersinia* and *Shigella*. The bacterial proteins are recognized by the corresponding *R* gene products — Pto, Rps2 or Rpm1 (which recognizes both AvrB and AvrRpm1) in the cytoplasm, or Bs3, which might be found in the nucleus. Upon recognition, defence responses are activated. Prf is required for Pto function, although it is not understood how it acts. Pti1 is phosphorylated by Pto, but it is not clear how this leads to activation of a defence response.

for-gene interactions be based on discriminating protein-for-protein interactions inside the plant cell? If they were, one would expect there to be a direct interaction between the bacterial *avrPto* gene product and the plant *Pto* gene product. Satisfyingly, in two papers published in *Science*^{1,2}, the Staskawicz–CEPRAP group (which isolated the *avrPto* and *Prf* genes) and the Martin group (which isolated the *Pto* and *Pti1* genes) confirm this prediction using the yeast two-hybrid system. Moreover, mutations of *Pto* or *avrPto* that disrupt the interactions in yeast abolish disease resistance in plants, and vice versa. Both groups also used *Agrobacterium* to transiently express *avrPto* in the leaves of plants that did, or did not, carry *Pto*. They observed an HR that was dependent on the simultaneous expression of *Pto* and *avrPto*, indicating that AvrPto must be delivered to the host-cell cytoplasm in order to be recognized.

Pto is 80% identical to the product of the closely linked *Fen* gene. Fen protein does not interact with AvrPto, so Fen was used in domain-swap experiments with Pto to define the region that is involved in recognition-specificity. Of the 321 amino acids that constitute Pto, the Staskawicz–CEPRAP group mapped the interacting region to amino acids 129–224, and Martin's group identified residues 190–209 as being critical. Remarkably, two of the conserved protein-kinase catalytic domains are at positions 182–184 and 202–211. In other words, the

specificity domains of the Pto protein kinase seem to be right in the middle of the catalytic domain. Moreover, a kinase-deficient Pto mutant did not interact with AvrPto, suggesting that autophosphorylation may be important in this interaction.

Although these data are significant they leave many questions open. Could AvrPto activate, or interfere with, interactions between Pto and Pti1 (or other proteins that interact with Pto)? And why is the cytoplasmic LRR protein Prf required for activation of the defence response? Could Prf, in fact, recognize a phosphorylated form of a plant protein that is an *avrPto*-dependent substrate for Pto, and then go on to activate a defence response analogous to that triggered by other cytoplasmic LRR genes? Whatever the mechanism, Pto must indeed be a discriminating protein kinase — a kinase with keen eyes for AvrPto.

How general might the delivery of *avr* gene products from the bacterial cytoplasm be? Two papers published in *Proceedings of the National Academy of Sciences*³ and in *Cell*⁴ shed more light on this question. In one study, which confirms and extends the work of Gopalan *et al.*¹⁸, Leister *et al.*³ used particle-bombardment to direct the transient expression of the *P. syringae* genes *avrB* or *avrRpt2* in *Arabidopsis* plants that had been mutated in either *Rpm1* or *Rps2* (see Table 1). They observed an HR only when both the functional *R* gene and the corresponding *avr* gene were expressed in the same cell. In

another study, Van den Ackerveken *et al.*⁴ used *Agrobacterium* to transiently express the *Xanthomonas* gene *avrBs3* in strains of pepper containing the *R* gene, *Bs3*. They observed a *Bs3*-dependent HR, suggesting that AvrBs3 and *Bs3* interact inside the plant cell — in this case, probably within the nucleus. However, the *Bs3* gene has not yet been isolated, so direct investigation of *Bs3* localization and the affinity of *Bs3* for AvrBs3 will have to wait.

The isolation of the plant *R* genes that confer resistance to bacterial pathogens has led to real insights into the nature and location of the molecular recognition events that underlie some gene-for-gene interactions. The new data allow a better understanding of bacterial pathogenicity mechanisms, and they render more plausible the notion that *avr* genes encode pathogenicity factors that the plant can evolve the capacity to recognize and respond to. But what is the function of the *avr* gene products and how do they perturb plant metabolism to favour the pathogen? For example, have the *avr* genes been selected to interfere with plant defence mechanisms or perhaps to enhance leakage of nutrients for bacterial growth? And why are some *avr* gene products targeted to the plant nucleus? Also, it is intriguing that several *R* genes that confer resistance to haustoria-forming fungal pathogens are of the cytoplasmic LRR type: could this mean that fungal products are recognized by cytoplasmic *R* gene products at, or near, the cell surface during the intimate association between the plant and the fungus at the haustorial interface? It is to be hoped that some of these questions will be addressed by the flood of interesting results from the study of plant–pathogen interactions which currently shows no sign of abating. □

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Creation in silicon

John L. Casti

The idea of creating a mechanical intelligence that can display human-like thought processes has been a dream pursued by the artificial-intelligence (AI) community from the dawning of the computer age. But since no one really knows how humans think, the test of whether a machine intelligence has the 'right stuff' has been based on the behaviouristic test, originally proposed by Alan Turing¹ in 1950, that the machine is intelligent — human-style — if a human interrogator can't tell the difference between interacting with a machine and with another human. Now a computer program put together by William McCune (Argonne National Lab., Illinois, USA) has found a solution² to a major mathematical problem that could be said to easily pass a Turing test for mathematical creativity, giving ammunition to supporters of the 'strong-AI' thesis that there is no intrinsic barrier to a computer thinking just like you and me.

The problem solved by McCune's program involves a conjecture in boolean algebras that a certain set of three equations constitutes a basis for such an algebra. The problem was first posed by Herbert Robbins in the 1930s, and was worked on by a number of mathematicians, including the famed logician Alfred Tarski. McCune's automated reasoning program, which is a general-purpose 'prover' of the truth or falsity of logical expressions, was given a statement of the Robbins problem, along with a few simple parameters to restrict the search, none of which was particularly focused on boolean algebras.

The program searched an infinite space of logical expressions and found a proof of the conjecture. In fact, the program found several different proofs of the conjecture, depending on various parameters, especially the maximum length of any expression encountered in the search process. The first proof of the Robbins conjecture took a total of about eight days of searching using three workstations.

Of course, computers have been used many times in the past to resolve mathematical conjectures. Probably the most well-known of these efforts was the work by Appel and Haken in 1976 to prove the famed four-colour conjecture³ (that four colours are enough to fill in any planar map, without having adjacent regions of the same colour). A similar exhaustive computer search by Clement Lam in 1988 showed the non-existence of projective planes of order 10. But in these cases the mathematicians outlined the proof beforehand, then wrote special-purpose software to check the large,

but finite, number of cases needed to establish the theorem. So there was a pretty good idea before the computation started as to how long it would take, and that an answer would be found.

In contrast, in his computer proof of the Robbins conjecture, McCune had no expectations of any sort that a proof would emerge when he started his program working on the problem. Moreover, what finally emerged was an explicit proof that can be checked by hand and by independent proof-checking programs. This contrasts strongly with the Appel-Haken and Lam computer proofs, whose correctness depends on the correctness of the software.

The solution of the Robbins conjecture was found by a program designed to reason, not to solve a specific problem. Whether this constitutes a 'creative' act or not is open to debate. But as Larry Wos, the supervisor of the computer reasoning project at Argonne says, "It's a sign of power, of reasoning power... We've taken a quantum leap forward". Wos predicts that this result may mark the beginning of a new era in mathematics, one in which mathematicians focus on producing interesting conjectures, leaving their proof or disproof to computer programs. These sorts of proofs by machine suggest that perhaps there is a finer line than one might have thought between mechanical search and creative acts.

But even if this line of work on automated theorem-proving does revolutionize mathematical practice, what does it mean for creative thought in general? Can we justify extrapolating a creative proof of a mathematical conjecture like the Robbins Conjecture into a belief that machines can construct equally creative works of art in other areas like poetry, literature or music? Or will they be confined to human-like acts of inspiration only in areas, like mathematics or chess, that are, by and large, outside the realm of everyday human experience and emotion? For years the strong-AI community has argued that there is no significant difference between the two. Anti-AI crusaders, such as John Searle of Berkeley, have stated that the gap is unbridgeable. McCune's work at least suggests that the whole issue may soon move from the realm of philosophical speculation to that of laboratory experiment. □

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Daedalus

Celestial torque

Conventional astronomy studies the stars and planets by their radiation alone. The gravitational fields of the planets extend to the Earth, but are ignored. Could a sensitive gravimeter detect them? At first sight, this seems impossible. Like a spacecraft, the Earth is in free fall among the planets. The gravimeter falls with it, and no force can be developed between them. However, free fall only masks the direct field of a heavenly body; its field gradient is still detectable. A satellite in free fall feels a torque from the gradient of the Earth's gravity — its slightly greater intensity on the side nearer the Earth. Indeed, gravity-gradient effects can be used to stabilize a satellite, keeping its long axis pointing at the Earth. Similar effects should be felt on the Earth due to masses in space. Olympic high-jumpers have been advised to wait till the Sun or Moon is directly overhead; its upward pull will gain them a extra fraction of a millimetre.

So Daedalus began to invent an earthbound gravity-gradiometer for detecting masses in space. It was a long rigid rod, freely suspended and equipped with delicate interferometric sensors. Nearby heavenly bodies would impose torques on it. As the Earth rotated, the intensity and angle of the torques would change, revealing the masses and positions of the bodies responsible. But he then realized that nature has already done it. The ocean tides are nothing but a natural gravity-gradient detector on a vast scale. They are, of course, dominated by the Moon and the Sun. Yet the nearer planets must raise tides of their own, typically a few micrometres high.

DREADCO physicists are now designing a tide-gauge to reveal them. A large vertical pipe will be anchored to the side of some atoll in deep mid-ocean. Its bottom will be so deep that wave action will not enter; its surface will respond only to tidal influences. A mirror floating on that surface will form part of a laser interferometer, or even a quantum tunnelling device, revealing the tidal flow with almost atomic precision. Unperturbed by daylight or cloud cover, it will open a new window on the Solar System. Since tidal effects intensify inversely as the cube of the distance, even satellites in low orbit might raise detectable tides. And should there be any of the proposed astronomical 'dark matter' in our neighbourhood, or some black asteroid on a collision course with Earth, it might elude our telescopes, but would show up unflinchingly on the interferometric tide-gauge.

David Jones

Carl Sagan (1934–96)

Astronomer and popularizer of science

The last third of the twentieth century will be remembered as the time when humans first ventured off Earth. A pivotal figure in this exploration of the Solar System, and the eloquent tour guide for our trip, Carl Sagan succumbed to pneumonia on 20 December 1996, after a two-year battle with myelodysplasia, a bone-marrow disease.

Sagan, educated in the humanities, physics, astronomy and genetics, chose to work in the nascent fields of exobiology and planetary science, starting under the pioneer Gerard Kuiper. His publications, more than 600 in number, spanned a remarkable breadth of fields. The possibility of life elsewhere was his scientific passion, and much of his work touched on some aspect of this, often by pointing out the harshness of our own surroundings.

In his mid-twenties, Carl argued that Venus's intense microwave emissions were thermal radiation from a planet heated by the greenhouse effect of its dense carbon-dioxide atmosphere. Limb observations taken by Mariner 2, the earliest successful interplanetary mission, supported his model. Carl and his first graduate student, the late Jim Pollack, suggested that Mars's changing surface features, which had led to speculations a century earlier that the planet might be habitable, could be produced by wind-blown dust. Mariner 9, the inaugural orbiter of another planet, found shifting dune fields.

Carl was among the first to point out the 'faint early Sun' problem in Earth habitability. Following experiments seen as a student at the University of Chicago, Carl, along with Bishun Khare, former students Chris Chyba and the late Reid Thompson, elucidated prebiotic conditions in the Solar System by zapping various mixtures of common gases with different energy sources to synthesize organic molecules of remarkable complexity. The resulting 'tholins' were good spectroscopic matches for organic-rich outer-Solar-System bodies and cometary dust. Perhaps the most poignant aspect of Carl's death is that the major questions concerning life elsewhere are likely to be answered soon. What are the biochemical pathways to life? How far have protobiological processes advanced on Mars, Europa and Titan? How common are extrasolar Earth-sized planets and what is their nature?

Carl's first book, written jointly with the



distinguished Soviet astrophysicist I. S. Shklovskii, laid out the physics and philosophy behind the search for extraterrestrial intelligence. The LAGEOS, Pioneer and Voyager spacecraft carried messages from Carl, Frank Drake and others, intended ostensibly for any

extraterrestrials who might happen upon the craft; the real purpose (well achieved) was to advertise to other humans that our species had begun to visit the stars.

Carl was not an aloof academic, but frequently entered the public-policy arena over issues such as the arms race, asteroid hazards, and environmental concerns such as the greenhouse effect and the ozone layer. The nuclear winter, Carl's most provocative investigation, developed with Pollack, Rich Turco, Tom Ackerman and former student Brian Toon, proposed that a full-scale conflagration would loft dust into the atmosphere (later Paul Crutzen added smoky firestorms); the resulting clouds could cool the Earth for decades, with consequences far more devastating than the original blast. The size of the effect, even its sign, remains controversial, but the failure of national security agencies to imagine this horrendous outcome highlighted the limitations of previous models.

Sagan enormously influenced the direction of the early US planetary programme, not so much in mission details (although he was active in planning the Mariner, Viking, Voyager and Galileo flights and interpreting their results), but through the publicity that he brought these enterprises and through his access to policy-makers. He was an unwavering critic of NASA's manned space programme, including the Space Station, and a staunch advocate of unmanned planetary exploration. With Bruce Murray, Carl founded the 100,000-member Planetary Society to involve average citizens in this great adventure and, when needed, to lobby the US Congress for its continuation.

Planetary studies was born as a scientific discipline three decades ago, and Carl was a midwife. He helped establish the Division for Planetary Sciences (DPS) of the American Astronomical Society, and

was one of its first chairmen. Early on, he took over the journal *Icarus*, editing it for 11 years. Most of all, Carl set the tone for the discipline, through his infectious enthusiasm about space exploration, his scientific generosity and his interdisciplinary interests. He enticed students and faculty, including myself, to join him in the voyages of discovery across the Solar System.

Carl's talent as a popularizer of science set him apart. A remarkably gifted writer, he was aptly called the poet laureate of science. As James Michener wrote when reviewing the book *Cosmos*, "His style is iridescent, with lights flashing upon unexpected juxtapositions of thought". *Dragons of Eden*, Sagan's ruminations on the evolution of the human brain, received a Pulitzer prize. All told, his books stood on bestseller lists for more than three years. At his death, he was co-producing the movie *Contact*, based on his novel, and the Omnimax film *Comet*.

The award-winning *Cosmos* television series, written with his wife-to-be, science author Annie Druyan, and Steven Soter, was seen by half a billion viewers worldwide. It was a visually stunning amalgam of anthropology, history, biology and astronomy, that showed how our changing perception of the Universe led to a new view of ourselves. In frequent appearances on late-night television, Carl's boyish good looks, puckish humour and charm overturned the popular perception of the colourless scientist.

Perhaps Carl's greatest public influence came through his columns in *Parade*, a Sunday newspaper supplement with a circulation exceeding 80 million. Here, often collaborating with Annie, he shared his exhilaration at nature's beauty and explained difficult scientific concepts, while simultaneously chiding the public for tolerating scientific charlatans. Because of his interest in exobiology and his visibility, Carl was often drawn into public debates about all manner of pseudoscience, from UFOs to parapsychology. With sharp wit, he argued vigorously for rationality and the scientific method, maintaining that the known world was fascinating enough — one need not look for extraterrestrials in every unexplained happening.

A first-generation planetary explorer of the first rank, Carl Sagan left a world that understood itself and its place in the Universe much better. What more can a scientist accomplish?

Joseph A. Burns

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Oldest record of a bisexual plant

We have discovered coalescent male and female gametangia in fossils of the earliest charophytes (one of the algal groups most closely related to higher plants) from the Devonian period. This provides the oldest evidence of bisexuality in plants. In contrast to the well-documented complex evolution of the female reproductive organs (the oogonia), these fossils demonstrate that, in charophytes, the structure of the male sex organs (antheridia) has remained relatively unchanged to the present day.

The extensive fossil record of the charophytes since the Late Silurian period is based almost exclusively on female fructifications¹. Material from Landeyran Quarry, near Cessenon on the southern margin of the French Massif Central, is unusual in that it exhibits reproductive organs of both sexes. Charophytes are commonly found in dolomitic rock sequences in this area, indicating past intertidal to supratidal conditions² and suggesting that the plants tolerated fluctuating salinity as does the extant genus *Lamprothamnium* J. Groves (Characeae). Associated conodonts indicate that the plants are 405 million years old (Early Lochkovian age; *Postwoschmidt* zone)³.

The Landeyran charophytes represent a single taxon. They consist of spheres 0.6–1.1 mm in diameter. The original cellular structures, diagenetically replaced by

haematite, show excellently preserved surface characters, especially numerous external moulds of antheridia (Fig. 1). Although the disposition of sexes is different from extant forms, these moulds show a similarly complex structure, that is, spherical bodies composed of imbricated triangular plates, each bearing a central pillar (manubrium) on its internal face where the spermatogenous filaments are inserted.

In the fossil material, the antheridia are borne by two ramified axes, symmetrical about the dorsoventral plane of the fructification. The oogonium, whose multicellular wall is visible in thin section, occupies all of the interior. This structure, consisting of an oogonium surrounded by fertile axes bearing antheridia, represents a utricle, similar to that developed in the Cretaceous Clavatoraceae *Perimneste* Harris. However, in the Cretaceous form the symmetry of the utricle is tripartite¹.

In its bilaterality, the Landeyran species is similar to the mid-Devonian *Pinnopotamen* Wang and Lu, in which the utricles also have two ramified, opposite branches at their surface, but lack antheridia. Despite these resemblances, it would be inadvisable to assign the Landeyran species to a definite group, in the absence of data on the disposition and number of gyrogonite cells. Heterogamy in land plants is attested by spore

tetrads displaying evidence of meiosis as early as the mid-Ordovician⁴, but distinct male and female gametangia are not known earlier than the Pragian Rhynie Chert⁵, five million years younger than the Landeyran occurrence. With regards to bisexuality, the oldest land plants bearing both male and female reproductive organs are the Late Devonian lycophytes⁶. So the Landeyran charophytes represent the oldest record of heterogamy and bisexuality.

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Blindsight in normal subjects?

Blindsight¹ is the condition in which patients with damage to the visual cortex of the brain nonetheless have residual visual sensitivity² in a subjectively blind part of the visual field. Kolb and Braun report³ an intriguing parallel to blindsight in normal subjects, who were briefly shown a pattern in which part of one quadrant of the display had a contrasting texture, which 'popped out' from the rest of the pattern. When this texture pattern, viewed by one eye, was masked by a complementary pattern in the other eye, the subjects' ratings of subjective confidence in their ability to detect the texture contrast showed no correlation with their success rate, whereas in the unmasked condition their confidence was highly related to their success rate.

We have reproduced Kolb and Braun's procedure as closely as possible using target quadrants defined by orientation, but did not find blindsight. Performance was better in the unmasked than in the masked condition, and there was a high correlation of confidence with success in both cases. When we altered the exposure durations to obtain comparable performance in the two conditions, success and confidence were still highly correlated (Fig. 1; Table 1).

Observers could identify the unmasked target after exposures of about 100 ms (followed by a post-stimulus mask), and two of the subjects (A. J. S. M. and J. L.) could also

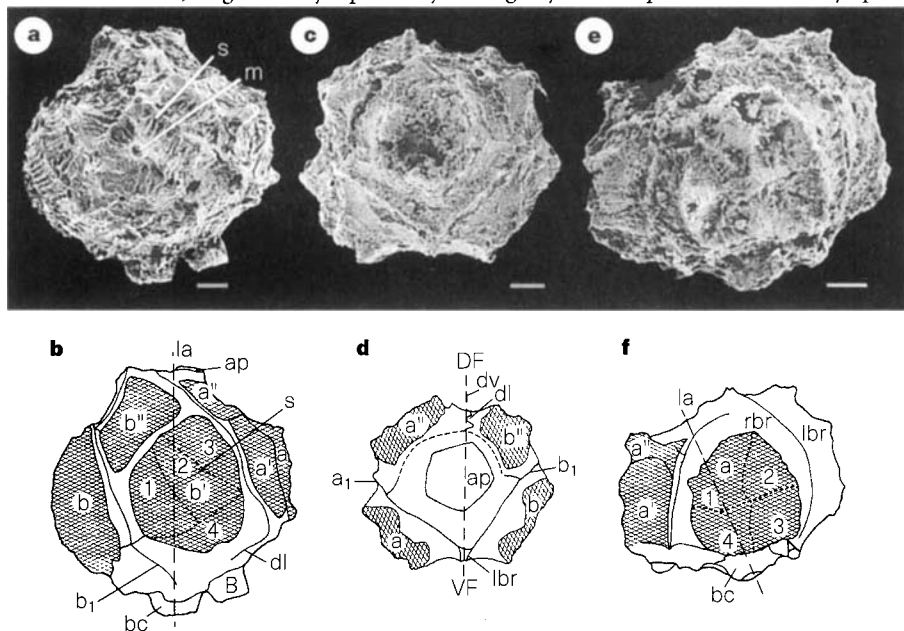


Figure 1 Utricles of a Devonian bisexual charophyte. **a**, Lateral view, showing the left branch bearing three antheridia and **b**, a reconstruction. **c**, Apical view and **d**, a reconstruction. **e**, Lateral view of the right side, showing lower large antheridial imprints borne on the right lateral branch and the ventral bractea, and **f**, a reconstruction. **s**, antheridial shield; **m**, manubrium scar; **A**, basal cell of the right branch; **a**, right branch; **a'**, **a''**, lower, middle and upper right antheridia, respectively; **B**, **b**, **b'**, **b''**, same nomenclature for the left branch; **ap**, apical pore of the gyrogonite; **bc**, basal cell; **DF**, dorsal face; **dl**, dorsal line; **dv**, dorsoventral symmetrical plane; **la**, longitudinal axis; **lbr**, left bractea; **rbr**, right bractea; **VF**, ventral face; 1–4, the four shields of the left antheridium. Scale bars, 100 μ m.

Heritabilities and paradigm shifts

In his influential book *The Structure of Scientific Revolutions*, Kuhn¹ described scientific endeavour in terms of occasional revolutionary shifts from one favoured paradigm to another. Although research is not totally uncritical between revolutions, currently popular explanations can be accepted too readily. Here, we provide a quantitative example from evolutionary biology.

Female choice of ornamented males has been hotly debated over the past 20 years². The 'good genes' school claims that ornaments are honest indicators of male viability, allowing choosy females to gain through enhanced offspring viability³. The 'fisherian' school argues that male ornaments evolve in a runaway fashion once females have started to favour ornamented males, and the ornaments convey no fitness benefits to the offspring. Between 1981 and 1986, orthodox theoretical models⁴ excluded the possibility of any additive genetic variance allowing females to 'shop for good genes'. However, new models that appeared between 1986 and 1988 indicated that ornaments can evolve as honest viability indicators⁵, and the previously suppressed good-genes theory was no longer considered naive.

Pomiankowski and Møller⁶ found high heritability estimates from a survey of the available data, which supported the underlying assumptions of good-gene models. But we have found a remarkable change in the data since 1988 (Fig. 1). The average heritability for ornaments reported before 1988 was 0.37 (s.d. = 0.14, $n = 10$), but subsequently rose to 0.67 (s.d. = 0.34, $n = 24$; Mann-Whitney rank test, $z = 2.29$, $P < 0.05$). The higher mean reflects a simultaneous increase in the variance of the estimates (for the increase in variance $F = 5.56$, $P < 0.01$). There were no significant changes in the mean heritability estimates for nonsexual traits (until 1987, mean = 0.40, s.d. = 0.19, $n = 9$; since 1988, mean = 0.53, s.d. = 0.20, $n = 9$; $z = 1.41$, $P > 0.10$). Additionally, there was no increase in the coefficient of additive genetic variance for the sexual traits ($z = 0.66$, $P > 0.10$). This index has been advocated as a preferred index⁶ only recently. It seems possible, therefore, that the changing heritabilities reflect publication biases rather than any changes in the features of the organisms.

These chronological changes in heritability estimates may reflect a Kuhnian paradigm shift, characterized by relatively conservative attitudes during the normal phase and a more cavalier approach during the revolutionary phase. Heritability estimates derived from small sample sizes are

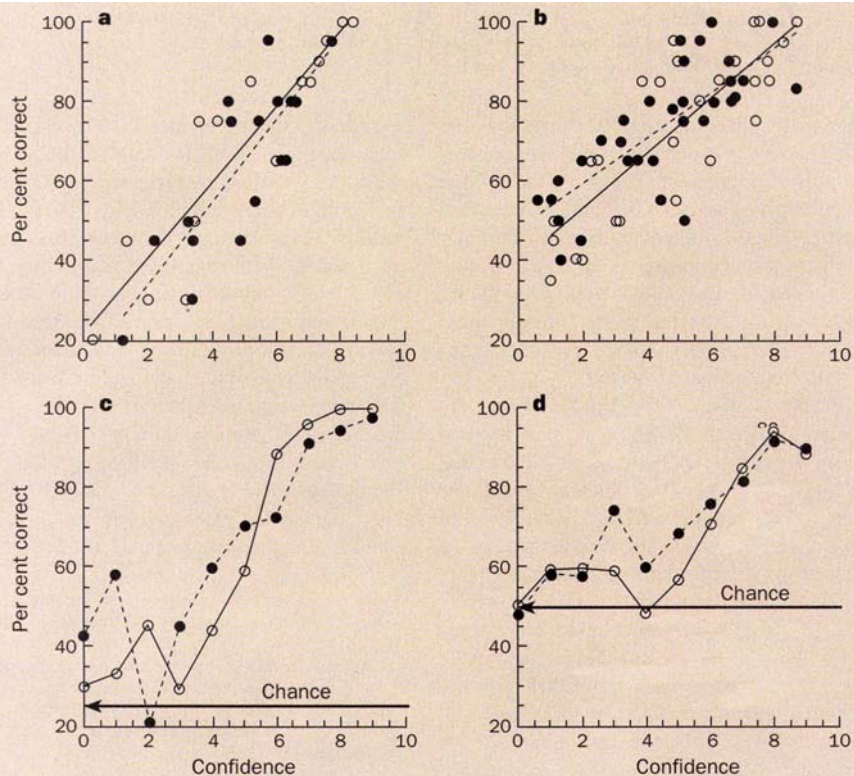


Figure 1 Correlation between success and confidence of success. Each point in a and b represents the performance of an individual subject at a particular exposure duration, in either the masked (solid symbols) or the unmasked (open symbols) condition. Linear regressions are shown (masked condition, broken lines; unmasked condition, solid lines). All correlation coefficients are high (a, 0.84, 0.92; b, 0.77, 0.92; masked, unmasked). In experiment 1 (a) the subjects were the three authors and masked and unmasked trials were randomly interleaved. In experiment 2 (b) the two conditions were presented separately and the subjects included three naive observers. Further observations were made of one other experienced subject (D. I. A. M.) in the masked condition alone (see Table 1). Details of the methods are available upon request from M. J. M. c, d, Replotting of the data from a and b, respectively. Each point represents the mean for all of the subjects, collapsed across exposure duration. Irrespective of duration, confidence is still a good predictor of success rate in both unmasked and masked conditions.

identify the target after similar exposures in the masked condition. They reported that the target did not appear to differ in orientation from the background (inconsistent with the explanation that suppression of one eye might be the cause) but that it did seem to differ in brightness or depth (possibly resulting from false stereo matching).

The other subjects required longer exposures to see the target in the masked condition. J. A. S. also saw the target as being defined by brightness or depth, whereas M. J. M. and D. I. A. M. saw a line surrounding the target. We presume that these verbal reports are post hoc rationalizations

for the segmentation of the image without an orientational cue. Clearly, the experience, although vague, is not unconscious.

Kolb and Braun's subjects were instructed to use the full confidence scale, irrespective of their absolute sense of certainty. If they were reluctant to use the vague cues in the masked condition as evidence for a high-confidence judgement, they may have decided to produce ratings randomly, with the result that Kolb and Braun observed. It is otherwise difficult to understand why they claimed to be highly confident: the opposite of blindsight. We conclude that blindsight in dichoptic displays is difficult to replicate. The motion transparency display used by Kolb and Braun may be more robust.

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Table 1 Success–confidence correlations

Subject	Unmasked	Masked
M. C.	0.99	0.77
F. F.	0.76	0.97
M. J. M.	0.90 (0.96)	0.99 (0.92)
A. J. S. M.	0.95 (0.98)	0.99 (0.94)
J. A. S.	0.87 (0.90)	0.91 (0.85)
J. L.	0.89	0.73
D. I. A. M.	–	0.95

Correlation between the per cent correct scores and the mean confidence rating. Figures in parentheses show results from experiment 1.

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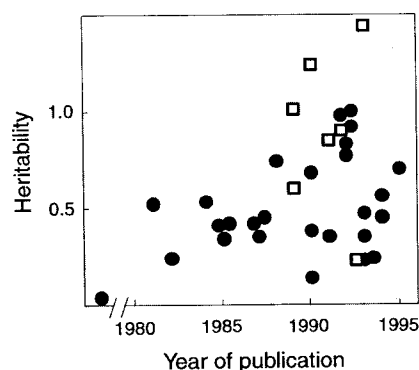


Figure 1 Heritabilities of secondary sexual characters listed in ref. 6 plotted against the year of publication. Open squares, studies with relatively small sample sizes, are based on the offspring of fewer than 20 males. Because the choice of the cutting year (1988) is not unambiguous we ran a series of simulations in which we randomized 1,000 times the 34 heritabilities to 34 publication years and checked all the cutting points (ensuring that the smaller data set had at least 10 observations) and took the largest difference in means and largest ratio in variances. This procedure was repeated 100 times to obtain error estimates, and both the observed differences in means ($P = 0.0260$, s.e.m. = 0.0004) and in variances ($P = 0.0052$, s.e.m. = 0.0003) are larger than their random expectations.

statistically significant only if the estimate is close to unity. Seven parent-offspring studies with fewer than 20 fathers, published between 1989 and 1993, have higher heritability estimates (mean = 0.90, s.d. = 0.40) than those of the remaining studies with larger sample sizes (mean = 0.50, s.d. = 0.25, $n = 27$, $z = 2.36$, $P < 0.05$). Although none of these studies is wrong, they do not represent a random sample of estimated heritabilities. Perhaps people who have obtained estimates from small sample sizes were unwilling or unable to publish their results during the time when the good-genes model was unpopular, whereas such data were more readily published during the 'revolutionary' phase of the acceptance of this model.

Ironically, heritabilities of ornaments do not solve the initial controversy between the fisherian and handicap models⁷ because both require some heritability for the ornaments. The essential difference is that female choice for good genes can occur only if ornaments are honest signals reflecting viability genes. These relationships have not been fully explored empirically, but many innovative studies have emerged^{12,6}.

Research on sexual selection by female choice appears to have followed Kuhn's paradigm-shift principle, at least to some degree, even if the result has been a synthesis⁸⁻¹⁰ rather than a replacement of ideas. Further, our analysis reveals the risks of relying solely on statistically significant results in the decision process of publishing

empirical research. Perhaps we should show more interest in the appropriateness of the study methods, sample sizes, powers of statistical tests and confidence intervals.

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Almost airborne

Insect wings appear to have evolved from articulated gills of aquatic ancestors^{1,2}. Possible remnants of intermediate stages in this transition occur in modern stoneflies that use non-flying aerodynamic locomotion to move across water surfaces. Such wing-propelled movement is a feasible setting for elaboration of wing size and the development of a powerful flight motor (muscles, neural patterns and wing articulations)³⁻⁵.

However, the mechanical requirements of surface locomotion are not as strict as those of flying. Flight requires dynamic control of body orientation⁶⁻⁸ and generation of sufficient lift to achieve full aerodynamic weight support. Given these mechanical challenges, how might surface-skimming insects have made the final transition to true aerial flight? Here we report that stoneflies of the genus *Leuctra* (Leuctrinae; Nemouridea, Plecoptera) use a form of locomotion, mechanically intermediate between skimming and flying, thus demonstrating an advanced stage on the continuum of two-dimensional surface locomotion that may have led to insect flight.

Adult *L. hippopus* and *L. sibleyi* emerge in spring when air temperatures are highly variable. Although otherwise relatively proficient flyers, on contact with the water surface at air temperatures below 13 °C, *Leuctra* stoneflies curve their body into a stereotypical 'S'-shaped posture, raising their fore- and middle legs while they flap their wings and thereby move across water. Maintaining a nearly vertical body orientation permits wing motion over a dramatically increased arc (nearly 180°) compared with surface skimmers that maintain water contact with all six feet and for whom the underlying water restricts wing-beat amplitude to 90–110° (ref. 3). Excessive forward or rearward pitch is controlled by briefly touching the middle feet or tip of the abdomen down on the water such that a stable, upright body position can be maintained over many wing-beat cycles. The risk

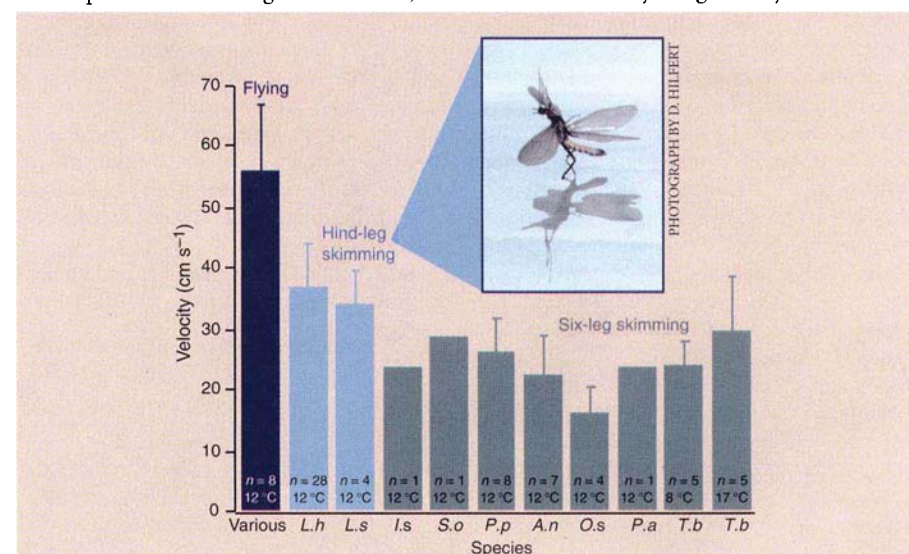


Figure 1 Inset, hind-leg skimming by *L. hippopus*. Photograph has been digitally enhanced. Histogram, velocities (± 1 s.d.) attained by stoneflies during flight, hind-leg skimming and conventional skimming (continuous six-legged water contact). n , number of individuals sampled (see ref. 3 for methods). 'Various' refers to individuals that took off and flew just above the water (*L. sibleyi* ($n = 3$), *Ostrocerca albidipennis* ($n = 2$), *Amphinemura nigrutta* ($n = 2$), *Sweltsa onkos* ($n = 1$)); *L.h.*, *L. hippopus* (Leuctrinae); *L.s.*, *L. sibleyi* (Leuctrinae); *I.s.*, *Isoperla* sp. (Perlodidae); *S.o.*, *S. onkos* (Chloroperlinae); *P.p.*, *Paranemoura perfecta* (Nemouridae); *A.n.*, *Amphinemura nigrutta*; *O.s.*, *Ostrocerca* spp. (Nemouridae); *P.a.*, *Paracapnia angulata* (Capniidae); *T.b.*, *Taeniopteryx burksi* (Taeniopterygidae; data from ref. 3). Note that surface skimming is widespread among the Plecoptera, contrary to previous suggestions⁹. A platform-independent QuickTime movie of hind-leg skimming is available at <http://cac.psu.edu/~jhm10>.

of injury during attitude control on water surfaces is minimal compared with that during gliding or crude flapping (the traditional model of insect flight evolution)⁸. Hind-leg skimming thus demonstrates how 'flight-ready' wing-beat kinematics and the ability to produce lift, thrust and the rudiments of attitude control might have evolved in a relatively safe setting before aerial flight in insects.

Leuctra stoneflies using hind-leg skimming attain speeds about 1.4 times faster than a taxonomically diverse sample of stoneflies that maintain water contact continuously with all six legs (Fig. 1). Higher speeds for hind-leg skimmers cannot be attributed to differences in body size (mean body lengths of *L. hippopus* and *L. sibleyi* are 6.5 and 5.0 mm respectively; the range for the other species tested is 4.5–8.0 mm; ANCOVA using body length as a covariate yields means of 36 cm s⁻¹ for hind-leg versus 25 cm s⁻¹ for six-leg skimmers; $P < 0.0001$).

Stoneflies tested at an air temperature of 12 °C can occasionally rise just above the water surface and fly. Velocity measurements of eight such events show that breaking free of the water and flying results in another 1.5-fold increase in forward speed compared with hind-leg skimming (using body length as a covariate for ANCOVA yields means of 56 versus 36 cm s⁻¹; $P < 0.0001$). Insects on water surfaces are exposed to predators, are displaced downstream by flowing water, and must often make headway against the wind, therefore faster skimming should generally enhance survival. Hind-leg skimming may be a favourable evolutionary transition from conventional skimming, with flight more favourable still. The various forms of surface locomotion used by modern stoneflies thus provide a detailed model for incremental improvements in aerodynamic locomotion that may have occurred during the evolution of flying insects from their aquatic ancestors.

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Nitrification in Antarctic soils

Marshall¹ found pollen from trees (*Nothofagus* spp. and *Podocarpus* spp.) and the spores of several fungi not normally native to Antarctica in air samples collected at Signy Island in the maritime Antarctic. Their presence was associated with a specific weather pattern, occurring with an estimated mean annual frequency of 1.5, that allowed wind-borne transfer of exotic biological particles to Antarctica from South America. As Marshall pointed out, such events provide potential mechanisms for organisms to extend their ranges into Antarctica. These observations apparently indicate that Antarctica is not a continent in biological isolation. We suggest here that bacteria may have been dispersed throughout Antarctica in a similar way.

We have estimated the numbers of nitrifying (chemoautotrophic, NH₄⁺-oxidizing) bacteria, and determined the short-term potential rates of nitrification (using the rate of NO₂⁻ accumulation²) at 12 °C (typical summer surface soil temperature), in seven different soils collected from continental and maritime Antarctica during the 1994–95 Antarctic summer (Table 1).

Nitrifying bacteria were detected in all except two soils (4 and 5). In soils 1, 2, 6 and 7 the most probable numbers of nitrifiers³ were low compared with temperate soils². The most probable numbers of nitrifiers and the potential rate of nitrification in soil 3 were high and similar to those in temperate soils with high nitrification rates². The potential nitrification rate in the other soils was either very low or not significant.

Soil 3 contained guano from an ancient penguin colony, as shown by our obser-

vation of remnants of Adélie penguin (*Pygoscelis adeliae*) feathers and the fact that it contained substantially more nitrogen than five of the other six soils. The fact that there were inactive nitrifiers in four soils indicates their widespread distribution but that soil and/or other environmental conditions restricted activity and, therefore, proliferation.

Nitrifier activity in soil 3 was apparently related to the source of nitrifiable nitrogen. The temperature optimum for potential nitrification in this soil was between 22 °C and 25 °C, with the rate at 7 °C being less than 15% of the maximum, whereas that at 35 °C was 40% of the maximum. The relatively high temperature optimum for nitrification in soil 3 indicates that the nitrifiers were probably not of polar origin.

In the light of Marshall's demonstration¹ of wind-borne dispersal of pollen and spores to Antarctica, it seems highly likely that there might be simultaneous dispersal of bacteria. If such dispersal of bacteria occurs, nitrifiers could be deposited on soils both with and without nitrifiable nitrogen. Our observations indicate that environmental conditions in terrestrial Antarctica, rather than its geographical isolation, may determine the activity of particular groups of bacteria.

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Table 1 Number of nitrifiers, potential nitrification rates and nitrogen content of Antarctic soils.

Soil	Log ₁₀ MPN nitrifiers (per g soil)	Potential nitrification (nmol g ⁻¹ h ⁻¹)	Total soil nitrogen (µg g ⁻¹)
1	2.38 (2.08, 2.67)	7.1 (0.58)	90 (4.2)
2	3.15 (2.86, 3.44)	2.5 (0.75)	250 (28)
3	4.54 (4.25, 4.83)	15.6 (4.92)	310 (50)
4	ND	2.7 (2.67)	7 (0.7)
5	ND	1.6 (4.02)	38 (9.2)
6	2.38 (2.08, 2.67)	1.3 (0.71)	37 (2.1)
7	-0.70 (-1.00, -0.40)	1.5 (1.04)	74 (5.9)

Soil 1 was from Jane Coll, Signy Island in the South Orkney Islands (62° S, 45.5° W); soils 2–7 were all from sites on Alexander Island in continental Antarctica; soils 2 and 3 were from Rothera (67.5° S, 68° W); soil 4 was from Ablation Valley (71° S, 68° W); soils 5 and 6 were from Fossil Bluff (71.3° S, 68° W); soil 7 was from Ares Oasis (72° S, 68° W). The most probable numbers (MPN) of ammonium-oxidizers were determined with five replicates at each 10-fold dilution. Values in parentheses are 95% confidence limits³. ND, none detected. The potential nitrification rate is the rate of NO₂⁻ accumulation in the presence of 0.5 mM NH₄⁺ and ClO₃⁻ (which inhibits NO₃⁻ oxidation²) at 12 °C, per g soil per hour. The values for nitrification and total nitrogen are the means of three and two replicates, respectively, and the standard deviations are shown in parentheses.

When superstition displaces science

Behind the Crystal Ball: Magic and Science from Antiquity to the New Age

by Anthony Aveni

Random House: 1996. Pp. 406. £20, \$27.50

Martin Gardner

Recent decades have seen an astonishing upsurge of popular infatuation with fringe science and the paranormal. A hundred years ago, no newspaper in the United States carried a horoscope. Today almost every paper except *The New York Times* has such a feature. A recent US president and his wife, Ronald and Nancy Reagan, were firm believers in the influence of stars on human events. Polls show that about half of all Americans believe in Satan, angels, demons, extrasensory perception, precognition and alien spacecraft invading our skies.

How does this compare with people's beliefs in earlier ages? It is the opinion of Anthony Aveni, in his entertaining history of Western occultism, that until the rise of modern science a belief in magic was rational, mainstream common sense. Not until after Galileo and Newton was such magic demoted to the fakery of stage conjuring. This shift surely has occurred among those knowledgeable about science. But has it also taken place among the masses whose knowledge of science could be scribbled on a postcard?

Two-thirds of Aveni's book can be seen as a skilful summary of Lynn Thorndike's multi-volumed *History of Magic and Experimental Science*, and one-third as taking up where Thorndike left off. The volume is amusingly illustrated, its bibliography extensive, and its facts carefully documented by footnotes. No recent popular history of occultism has covered such a wide swath.

Aveni begins with the "magic" of ancient Egypt and Mesopotamia, followed by Greece and Rome. After a colourful romp through the Middle Ages and Renaissance, he turns to the scientific rubbish of the nineteenth century. There are excellent accounts of the origin of modern spiritualism in the toe rappings of the Fox sisters, the levitations and other paranormal feats of the medium D. D. Home, Madame H. P. Blavatsky's theological twaddle, and the worldwide spread of phrenology.

Chapters on twentieth-century "magic" include the emergence of parapsychology, the spoon-bending of Uri Geller, crystal power, UFO abductions, dowsing, channelling, near-death experiences, alternative medicines and many other marvels.

Although Aveni, a professor of astronomy and anthropology at Colgate University in Hamilton, New York, clearly does not

believe any of the humbug he writes about, for some reason he feels obliged to be objective, to give balanced accounts of everything. "My goal will not be to attack alternative ways of perceiving reality," he writes. "I have no desire to pronounce all magic superstitious flotsam — science gone awry. Nor do I wish to demystify magic and reduce it to a set of explanations that are inferior to my own scientifically trained way of understanding the world."

I find it incredible that an astronomer would not wish to say that belief in the magic he covers so well in his book is inferior to his beliefs as a scientist. It is this tinge of cultural relativism — springing no doubt from Aveni's anthropologist's hat — that, to an old debunker like me, detracts from the value of his history. For example, although Aveni surely knows that Ted Serios, the Chicago bellhop who projected his thoughts on to Polaroid film, is not a genuine psychic, he cannot bring himself to say this outright. Instead, after mentioning that magician James Randi can duplicate all the feats of Geller and Serios, he adds: "Does this prove that Geller and Serios are fakes? In other

words, does the possibility of fraud mean that it actually had occurred?" Later, Aveni soberly considers the theory of some parapsychologists that spoon-bending can result from the mind using quantum laws to alter metal.

Aveni certainly agrees with Houdini that Home was "a hypocrite of the deepest dye" yet, after publishing Robert Browning's damaging account of a Home seance, he has to "balance" it with Mrs Browning's high praise of Home. One longs for Aveni to abandon his curious notion that science and magic are somehow equally valid ways of seeing the world.

I would have liked to see some mention of Chinese and Hindu astrology, each of which is based on star patterns entirely unlike those of Western astrology. (If one is right, the other two are wrong.) His discussion of alchemy misses the intense labours of Isaac Newton. A chapter on anthropology deals with the sorcery of primitive cultures, but is silent on Margaret Mead's naive beliefs in magic and UFOs, and on modern anthropologists and sociologists who are deep into the paranormal. There is nothing about St



Dabbling in the world of the waterfowl

The Wood Duck of North America (left) and the Mandarin of Asia are closely related species that have been described as the world's most beautiful wildfowl. *The Wood Duck and the Mandarin* (University of California Press, \$45, £28) follows the birds as

they court, search for nesting places, raise their young and migrate between their breeding and wintering grounds. The authors, Lawton L. Shurtleff and Christopher Savage, have studied the birds in the wild for many years.

Augustine's well-reasoned attacks on astrology, or the bashing of psychic charlatans by the Greek satirist Lucian.

I think the gulf between the opinions of intellectuals and the superstitions of the populace was not much wider in past ages than it is today. Is a person who takes homeopathic remedies, knowing they contain not a single molecule of the original substance, much different from a mediaeval peasant who tried to cure an ailment by drinking the blood of a frog? Is there much difference between those in past centuries who had visions of the Virgin Mary, angels and devils, and today's devout Catholics who have similar visions? Every time there is a report of a Mary statue that weeps or bleeds, the church is invaded by hordes of the faithful eager to witness the miracle.

Tens of thousands of Americans now regularly telephone 'psychics' for information. Do they differ from ancient Romans who sought similar advice from the patterns of animal livers and entrails, or from the voices of oracles?

For evidence that the gap between superstition and science is almost as wide today as it ever was, compare the number of books on the 'New Age' shelves of bookshops with the number of books about science. Only the kinds of superstitions have altered. And in some cases, such as astrology, not even that has changed. □

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One of the greats

The Life and Legacy of G. I. Taylor

by George Batchelor

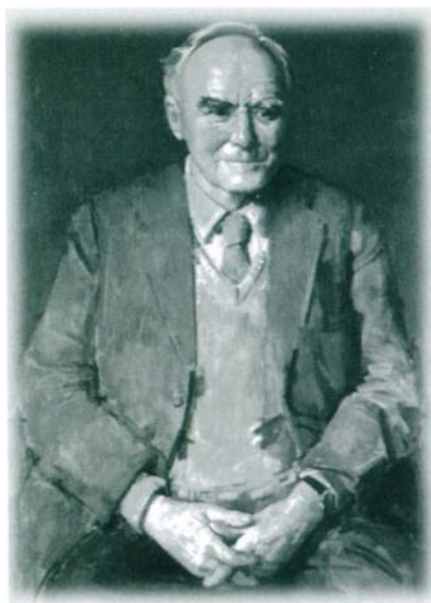
Cambridge University Press: 1996. Pp. 285.

£45, \$75.

J. C. R. Hunt

Much of our current understanding of the mechanics of fluids is owed to Geoffrey Ingram Taylor (1886–1975) and his two great peers, Theodore von Kármán and Ludwig Prandtl. Working from first principles of newtonian mechanics (as expressed in the differential equations of Navier and Stokes), classical thermodynamics, nineteenth-century mathematical methods and brilliantly insightful experiments, these three made many of the fundamental discoveries of phenomena and led theoretical development. In solid mechanics, Taylor's name is also one of a small number of 'greats', and the method of research has been similar. (The fields still share the same international congress every four years.)

George Batchelor, a research student of Taylor's between 1945 and 1948, and subsequently his colleague at Trinity College, University of Cambridge and editor of his collected papers, has written this scientific



Taylor: fundamental work on fluid mechanics.

biography especially, as he puts it, to help future students understand Taylor's greatness. He believes students should benefit from reading about his life and his approach to scientific research, in particular by using economical mathematical analysis, laboratory-scale studies and connections to practical problems. Older scientists, especially specialists in mechanics, will certainly enjoy this extensive account of their mentor, "G. I.", and his work.

The stimuli for Taylor's research in fluid and solid mechanics were initially practical. One was to reduce the danger to navigation in the North Atlantic from icebergs following the sinking of the *Titanic* — he was a member of the crew on the subsequent investigating voyage of SS *Scotia* — and others were the problems in aeronautics, structures and explosives in the two world wars, when he was a UK government employee and consultant. Taylor was also an early academic entrepreneur — he set up his own company in the 1930s to exploit his invention of the revolutionary 'CQR' anchor with its V-shaped blade that digs itself into the seabed when tension is applied.

Taylor's research on turbulence began with his measurements of the atmospheric humidity and temperature profiles using kites from the deck of SS *Scotia*, under the watchful eye of its grumpy captain. Relating the data to new mathematical theory, including early use of statistical correlations in fluid mechanics, he discovered some of the fundamental properties of turbulence and explored its different manifestations in the atmosphere, oceans and aeronautical and mechanical-engineering flows. He showed that all fluid turbulence exhibits some essential similarities at the smallest scales of motion — an idea that gained force in the 1930s and 1940s and was developed in

different ways by Lewis F. Richardson, von Kármán, Andrei Kolmogorov and others, including Batchelor.

But Taylor's formulae for the estimation of the transfer rates of momentum and heat by turbulent eddies have not had the same general acceptance. Batchelor describes how Taylor was critical of Prandtl's concept of a 'mixing length' model, an analogy with the kinetic theory of gases, because it does not reflect the difference between these two transfer processes. In fact Prandtl was aware of this point, as he made clear in *Essentials of Fluid Dynamics* (published in 1956), as are the many users of turbulence computer codes which are still largely based on Prandtl's concept. The users need, of course, to remember the kind of cautionary remarks Taylor made.

These studies led Taylor to his most fundamental and greatest fluid-mechanics paper, published in 1926, on the onset of instability and the resultant cellular pattern of flow that occurs in the narrow gap between cylinders when the difference in their rotation rates exceeds a certain value. This was the first experimental demonstration of the value of the eigenmode calculations of instability of viscous flows, a method later used for many practical problems, including aeronautics and plasma physics.

In the 1930s, Taylor pioneered the analysis of high-speed gas flows and shock waves around projectiles (which inspired Stanley Hooker, as a young researcher at Oxford and later as designer of the vital supercharger on the Spitfire engines). The experiences of the aerodynamicist Sir Arnold Hall and others of Taylor as a research supervisor show that he gave invaluable and kindly advice but remained somewhat detached.

Taylor's fluid-mechanics research, together with his solid-mechanics studies of plastic deformation at critical high-stress points, enabled him to provide invaluable consulting advice on offensive and defensive systems in the Second World War. This also led to striking new concepts which are still being worked on. In analysing explosions, he discovered the fundamental Rayleigh–Taylor instability, when an interface between two fluids is accelerated towards the less dense; and in helping to design a system for freeing airfields of fog using lines of heaters, he developed a theory for turbulent entrainment (not dissimilar to Prandtl's) that has subsequently become a basic modelling concept for environmental flows, cloud physics and oceanic modelling.

There were three elements to many of these wartime scientific investigations: field experiments on a huge scale; theories worked out at his home in Cambridge with occasional help from a Brunsviga calculator; and small laboratory studies at the Caven-dish. Perhaps Batchelor's admiration for Taylor's 'small science' is overdone

when he implies that only the last two elements were really necessary.

Taylor's mathematics are touched on in places, for example his use of the time-honoured method of similarity solutions. His approach was to find a convenient and tractable mathematical solution and not to worry too much about when it might be valid, or whether it was unique or even properly existed; he regarded such questions as pedantic or, if they were important, believed that they could easily be resolved by recourse to physical argument or experiment. However, as Norbert Wiener pointed out, Taylor certainly did introduce new mathematical thinking in the study of random processes. Although we can still learn much from his approach, the extensive and deeper use of computing has forced applied mathematicians and physicists to become more mathematical in their analyses of problems in mechanics.

Does the book indicate how Taylor's achievements related to his personality, his upbringing and the institutions where he studied and worked? Batchelor gives his views but also enough material for the reader to piece together his or her own. Taylor had a creative north London background, the son of an illustrator and educated at the nonconformist, intellectually self-confident University College School. He sailed down to the mouth of the Thames in a home-made boat as a teenager. This upbringing certainly seems consistent with the photographs of the confident young man who answered back to the captain of the *Scotia*; who requested his fiancée in the 1920s to read John Donne's poetry on the 'corporeal' aspects of love; who flew his own experimental plane in the First World War; and who in 1945, when in his 60s, was not afraid to publish his estimates of the strength of the Hiroshima atom bomb (to the distinct disapproval of the US authorities).

Batchelor emphasizes Taylor's lack of political and social opinions; but Taylor did have strongly held views about the world's rapidly growing population, a common view in his generation, and well known in circles connected with University College. Nevertheless, he was an enthusiast, at least, about some aspects of modern life, including, apparently, water-skiing at the age of 80!

My only complaint on reaching the end of this most enjoyable book is that there are not more stories about Taylor or about the factors and friends that influenced him early in his career; I hope that the search for correspondence about him will continue. This is a splendid book and it needed to be written by Batchelor, Taylor's great admirer and interpreter. □

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After the Ark

Noah's Flood: The Genesis Story in Western Thought

by Norman Cohn

Yale University Press: 1996. Pp. 150. \$30, £19.95

Martin Rudwick

Whenever science and religion are mentioned together, the image of perennial conflict continues to predominate, not only with the public but also among scientists. Yet the 'conflict model' has long been abandoned by historians of science as a gross oversimplification of a complex and fluid relationship. Abandoning stereotypes brings rewards, in opening the way for a more realistic understanding of the public uses of scientific knowledge — and not only of uses framed in religious terms.

Among points of potential interaction, the Flood in Genesis has had a historically unique status. Almost alone among the events that crowd the biblical narratives, and that have been endowed with human significance by religious traditions, the Flood was one that could be expected to

have left substantial physical traces. As a precious human record from the distant past, the story would have been used as an explanatory resource for understanding the natural world, even if it had not been privileged still more as a part of sacred scripture.

By being a putatively historical event, the Flood story belonged both to the world of religious meaning — at least for historical religions such as Judaism and Christianity — and to the world of physical explanation. Consequently, its role in the complex interaction between religious and scientific understanding has been quite different from that of, say, 'arguments from design'; for these, in forms both ancient and modern, are intrinsically ahistorical.

Norman Cohn, the author of *The Pursuit of the Millennium* (1957) and other important historical works, has now written an attractive brief survey of the fortunes and uses of the Flood story, ranging from ancient Mesopotamia to the equally alien territory of twentieth-century American creationism. On the way, he takes in the period of intense physical theorizing about the Earth in the seventeenth century, which

Diving to the depths in pursuit of history

Underwater exploration has a long history, as Jean-Yves Blot shows in his new book.

He writes: "In 1446 the Italian architect and writer Leon Battista Alberti searched Lake Nemi near Rome for traces of two great Roman vessels whose existence had long been part of local folklore, fuelled by the sporadic finds of fishermen." Alberti found pieces of wood from the wrecks which he thought dated from the era of the Emperor Trajan, who died in 117 AD. Blot brings the story up to date by describing the dangerous and exciting work of modern divers. The wreck of the Byzantine vessel *Yassi Ada I*, discovered at a depth of 36 metres in the Mediterranean, was found to hold a cargo of 900 amphorae (right).

Underwater Archaeology: Exploring the World Beneath the Sea is one of the latest titles in the excellent series of New Horizons paperbacks from Thames and Hudson (£6.95).



was of decisive importance in the origins of the modern Earth sciences.

Before he reaches that point, however, he deals with the ancient non-biblical Flood stories, explaining the huge impact of the clay tablets that revealed the Gilgamesh epic to the Victorians. He also mentions the rich repertoire of typological interpretation that was taken for granted in the patristic and mediaeval study of biblical texts — Noah and the Ark as the metaphors or 'types' of Christ and the Church, for example — which forms such a contrast to the rigidly literal methods of modern fundamentalists.

However, that literalism has its distant roots in the highly rationalistic activities of seventeenth-century scholars and naturalists — it would be grossly anachronistic to call them scientists — who set about calculating, for example, how the Ark could have contained the requisite pairs of all known animals. Similarly, the latest and trendiest scientific knowledge, such as of the motions of comets, was brought in to explain the physical causes of the Flood. But, rather than laughing or sneering at such work, we should recognize in it the exploratory spirit of modern science. As with more modern research, it was self-correcting: the theories, like the reconstructed Ark, burst at the seams, and their underlying assumptions were examined critically and then refined or rejected.

By the early nineteenth century, the extremely peculiar physical features that were attributed to the Flood could be metamorphosed conceptually with little difficulty into the traces of a vast glaciation. By then, the Flood/Ice Age was recognized as both ancient in human terms, yet geologically recent: the short span of human history was fully integrated into the huge timescale of geohistory.

Cohn's survey touches only lightly on such themes of overarching historical interpretation. For the most part it is a highly readable and reliable summary of what successive authors thought and wrote about the Flood, its physical traces and its religious meaning. His account is accompanied by a wealth of attractive illustrations, beautifully reproduced to Yale's customary standards. They are generally pertinent to the text but only loosely linked with it: Kircher's prosaic Ark rubs against a charming twelfth-century predecessor, with little regard for half a millennium's change of context.

Still, this is a book that is well worth a read by anyone with an interest in the historical roots of modern scientific study of the Earth. □

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Take a nature walk on the ocean floor

Oceanography: An Illustrated Guide

by C. P. Summerhayes and S.A. Thorpe
Manson Publishing: 1996. Distributed in the United States by Wiley. Pp. 352. £48, \$74.95 (hbk), £24.95 (pbk)

Peter G. Brewer

Ocean science in Britain has been through a period of great change, culminating in the creation of a new institute, the Southampton Oceanography Centre. This brings together previously separate research groups and ships in a fine new setting, and provides one of the world's major oceanographic research centres.

This book was written by the staff of the centre to mark the occasion. It is a guide, not an advanced textbook, and the stated purpose is to "express some of the delight" of being an ocean scientist, and to provide a description of the field "at a level at which a science undergraduate should have no difficulty in understanding". How well have they done? Very well indeed.

Written as a comprehensive collection of scholarly essays by diverse authors on important topics in the field, with excellent colour illustrations, the book holds together remarkably well. The editorial mandate must have been to use no equations, and only

the chapter on oceanic carbon chemistry manages modestly to get by this barrier. Nonetheless it would be a great mistake to regard this book as lightweight. The authors are experts, and whether the topic is the evolution of the lithosphere or of deep-sea animals there are densely argued presentations.

For the most part the presentation is up to date. The following subjects are all covered superbly in understandable terms: the ocean-climate connection, ocean-observing satellites, new discoveries of the extraordinary effects of iron enrichment of blue oceanic waters, and a synthesis of recent knowledge of hydrothermal activity and of the structure of ocean basins. And there is a wonderful nature walk on the ocean floor from Ireland to Florida with an educated guide at your side.

There are gaps: the molecular revolution in biology is not present in any way here, although it is providing dramatic advances in understanding at all levels. And palaeo-oceanography and microbiology are skimmed. There is an excellent historical chapter, and several applied science topics — instrumentation, marine resources, and scientific diving — that provide much of the lore well known to those in the field.

I could not teach a course solely from this book, but I would love to have it at my side when I do. It is a delight to dip into. □

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New in paperback

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Reviewed by V. Smil in *Nature* 379, 593 (1996).

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Episodic jets from black holes and protostars

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Energetic, highly collimated, episodic jets are observed in active galactic nuclei, binary stellar systems harbouring a black hole or white dwarf, and in association with stars in the process of formation. The common feature of these systems is that gas is fed onto the central object through an accretion disk. Time-dependent magnetohydrodynamical simulations show that magnetic fields anchored in the accretion disk can accelerate and collimate outflows into jets. For certain magnetic field configurations, episodic knots within the jet are produced.

There is now substantial evidence that jets in diverse systems are associated with gaseous disks^{1,5}. Such jets are powerful, with kinetic luminosities comparable to the radiative luminosity of the central source in some case⁶. Outflows are also long-lived; for protostars they persist for several hundred thousand years⁷ which is comparable to the star formation timescale. It is crucial to understand how accreting black holes⁸, stellar remnants or pre-main-sequence stars produce jets.

One unifying idea is that the detailed nature of the central body is itself unimportant; rather, its primary role in the jet phenomenon is in providing a deep gravitational potential well which is the ultimate energy source for the outflows. Gas in any astrophysical environment has some angular momentum so that, independently of how it is supplied to the vicinity of the central object, it will form a disk in keplerian motion around the centre. The removal of the angular momentum of the gas allows it to spiral slowly inwards through the disk and onto the central object, releasing gravitational potential energy as it goes. If the only torques on the disk are internal (for example, through magnetohydrodynamic (MHD) turbulence driven by magnetorotational instability^{9–13}), then angular momentum will be redistributed within the disk. The fraction of disk gas that loses angular momentum is accreted, releasing gravitational binding energy as heat which is subsequently radiated away^{14–16}. On the other hand, a number of steady-state MHD models^{17–23} of accretion disks have been constructed which rely on an external magnetic torque exerted on the disk by a jet or wind that can efficiently extract angular momentum. A significant fraction of the gravitational binding energy, instead of being liberated as heat, is converted into the mechanical energy of the jet. Thus, jets are powerful because they efficiently tap the gravitational potential-energy reservoir of the central object.

Here we investigate how jets are produced, not how jets interact with the environment on much larger scales. We use numerical algorithms to construct solutions to the time-dependent, nonlinear equations of axisymmetric ideal MHD to study outflows driven from the surfaces of an accretion disk. This allows us to relax the highly restrictive mathematical assumptions imposed by existing steady-state theories^{17,20,24–26}. Only time-dependent studies can follow the apparently universal, episodic, behaviour of jets^{7,27}. Numerical simulations of axisymmetric magnetized accretion disks in which the internal dynamics of the disk is followed find a rapid radial collapse of the disk. Although the highly twisted magnetic field generated in this process does push out a burst of matter as it ‘uncoils’^{28–30}, these models have not yet demonstrated that truly episodic ejection develops.

We adopt a different approach that allows us to isolate the physical processes involved in the formation of the jet for a well

defined set of conditions imposed at the base of the wind³¹. Accordingly, we present high-resolution, MHD simulations of the onset and collimation of outflows from the surface of a keplerian accretion disk around a central object for different initial magnetic configurations which provide the external disk torque. We present the results of 12 simulations which show that outflows either self-collimate into stationary, jet-like flows or remain episodic for the duration of the calculation. We have also found that for each of our initial magnetic structures, the rate at which matter enters the corona from the disk is an important factor in determining whether outflows are ultimately stationary or episodic. This implies that the internal dynamics of the disk ultimately may be crucial to understanding the operation of the outflow mechanism.

Model description

The numerical model for both types of simulations are solutions to the time-dependent MHD equations in cylindrical coordinates (r, ϕ, z) calculated with the ZEUS-2D code^{32,33} (except the adiabatic equation of state has been replaced with a polytrope). The model consists of a point mass representing a star or black hole, the surface of a surrounding accretion disk which is taken to have an inner radius r_i (this can be either the surface of the star, the magnetopause radius of a star with the disk, or the last stable Kepler-like orbit around a black hole), an initial disk corona which is in hydrostatic equilibrium within the gravitational field of the central object as well as in pressure balance with the disk below, and an initial magnetic field that threads the disk and corona. We set the initial toroidal field component B_ϕ and gas velocity everywhere in the corona equal to zero, and choose two initial magnetic configurations which are force-free. We avoid the use of a softening parameter for the (newtonian) potential^{29,31} and thus take particular care to establish the disk corona in numerical hydrostatic equilibrium so that there are no initial motions.

The keplerian accretion disk surface (the base of the corona at $z = 0$) provides fixed boundary conditions for the velocity at every disk radius; the rotational speed is keplerian v_K and as any radial inflow speed is expected to be tiny compared to this, we ignore it. Matter is introduced into the corona (active zones of the grid) from the disk at very small injection speeds v_{inj} ; for Figs 1–6, $v_{inj} \approx 10^{-3} v_K$ (one-hundredth of the disk’s sound speed). The disk’s toroidal magnetic field is assumed to scale with radius as²⁰ $B_\phi \propto 1/r$. The initial radial and vertical magnetic-field components in the disk are continuous with the field in the corona. There is no disk, rotation, or toroidal field inside the radius r_i .

All length scales and speeds in the simulations are measured in units of innermost disk radius r_i and the Kepler speed at that radius,

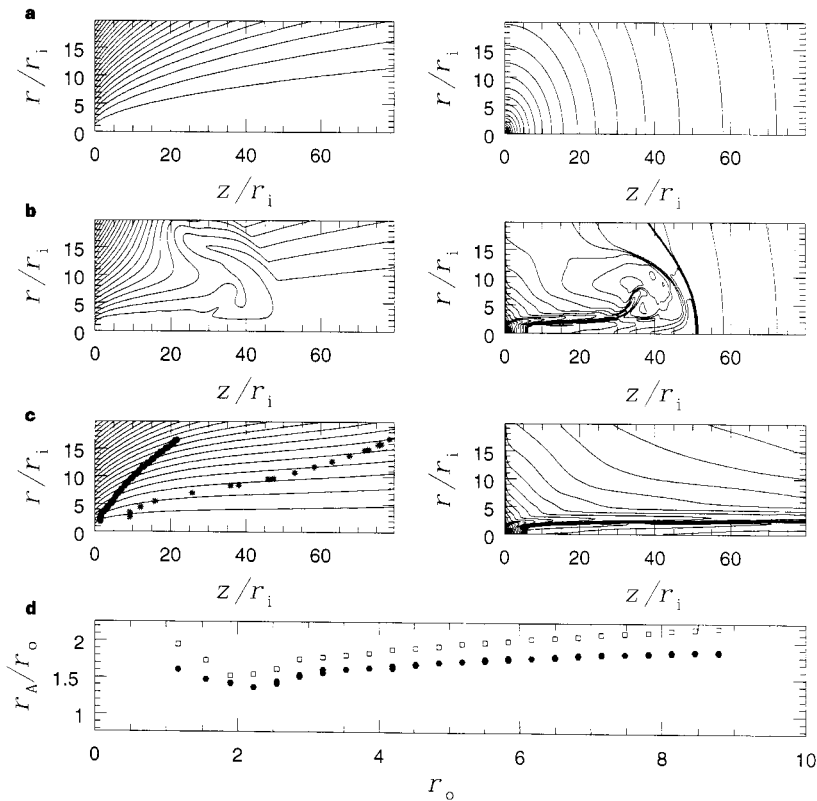


Figure 1 In all figures (Figs 1–6) the axis of symmetry is plotted horizontally and the disk surface, vertically; r_i is the innermost radius of the accretion disk. We use cylindrical coordinates (r, ϕ, z) . The gas has a polytropic ($\gamma = 5/3$) equation of state (γ is the polytropic index). The corona has a turbulent pressure proportional to the thermal pressure. The equilibrium density structure is $\rho \propto (r^2 + z^2)^{-3/2}$. Surfaces of constant magnetic flux are specified by the level surfaces of the scalar function A_ϕ where $\mathbf{B} = \nabla \times (A_\phi \mathbf{e}_\phi)$. The value of β at the base of the disk's corona falls from the innermost value of unity, to an outermost value (at $20r_i$) of 0.2 for our steady outflow simulation, and 10^{-3} for our episodic model. The remaining parameters are the square of the ratio of the Kepler speed to the sound speed, δ ; the ratio of the disk density to the coronal density, η_i ; and the disk's toroidal to poloidal field ratio, μ . For all figures (Figs 1–6), the injection speed is $v_i/v_K = 10^{-3}$; and $(\beta, \delta, \eta_i, \mu) = (1.0, 100.0, 100.0, 1.0)$. All the models are run at high spatial resolution

of (500×200) zones. We use inflow boundary conditions at the disk surface and open outflow conditions (that is, zero normal gradient) on the remaining boundaries (except along the axis of symmetry, coinciding with the disk axis, taken to be reflecting (that is, zero normal velocity)). **a**, The initial magnetic configuration (left panel) is the current-free 'potential field' solution; $A_\phi \propto (\sqrt{r^2 + (z_d + z)^2} - (z_d + z))/r$, where z_d is the disk half-thickness. The right panel shows the initial isodensity contours of the corona. **b** and **c**, The initial magnetic and density structure (the left and right panels respectively) at 100 (**b**) and 400 (**c**) inner time units, showing temporal evolution. In **c**, the location of the Alfvén critical surface (large dots) and of the fast magnetosonic surface (stars) is shown. **d**, Comparison of the Alfvén lever arm r_A/r_0 (the footpoint of each field line on the disk is r_0) for each field line in the simulation (large dots) with predictions of steady-state theory (empty squares).

$v_{K,i}$, so that results can be scaled to a central object of any desired mass. All timescales are measured in units of $t_i = r_i/v_{K,i}$. Typical conditions in protostellar or active galactic nuclei (AGN) disks are summarized in Table 1 where for the former we take³⁴ r_i to be 3 stellar radii and for the latter, 10 black-hole radii³⁵.

Our numerical simulations are governed by five parameters defined at r_i , the most important being the ratio of the thermal gas pressure to the magnetic field pressure at the base of the corona, β_i ; and the mass injection rate per unit area of the disk into the corona, $(\rho v)_{inj,i}$, where ρ is the density. The results in Figs 1–4 pertain to a domain of $(z \times r) = (80.0 \times 20.0)r_i$ in size, while those in Figs 5 and 6 pertain to a domain $(20.0 \times 10.0)r_i$ in size. As expected, the first stage of evolution in our simulations is a brief transient as a torsional Alfvén wave front propagates through the corona from the disk surface. The flow that develops in the next stage either relaxes to a steady-state pattern, or to an episodic outflow, as discussed below.

Steady-state outflow

We show in Fig. 1 a series of snapshots of the flow that illustrate the eventual realization of a stationary hydromagnetic outflow. In Fig. 1a, we plot surfaces of constant magnetic flux for an assumed

potential field configuration and density isocontours at the initial instant. The evolution of the two-dimensional magnetic field structure is shown in Fig. 1b and c, at 100 inner time units, and at 400 time units by which time the working surface of the jet has long left the end of our mesh. At time 100, the region behind the working surface of the flow has already achieved a cylindrically collimated state while the region ahead of the working surface still retains the original field structure. At time 400, the entire flow in our mesh has been collimated. Cylindrical collimation is predicted for steady MHD winds²⁶. There are three wave families and corresponding critical surfaces in MHD (in contrast to only one in ordinary hydrodynamics) and we plot two of them in Fig. 1c; the points at which the flow speed along streamlines is equal to the Alfvén speed (large dots), or the fast magnetosonic (FM) speed (stars). There is good agreement between our numerical results and steady-state theory for the position of the Alfvén surface as is shown in Fig. 1d. At the base of the outflow, the flux surfaces in Fig. 1c (or equivalently, the stream-lines) makes an angle of $\sim 55^\circ$ with respect to the disk across most of its surface. This geometry satisfies the condition, from steady-state centrifugally driven wind theory¹⁷, that the opening angle be less than 60° .

We plot in Fig. 2 the two-dimensional distribution of the toroidal

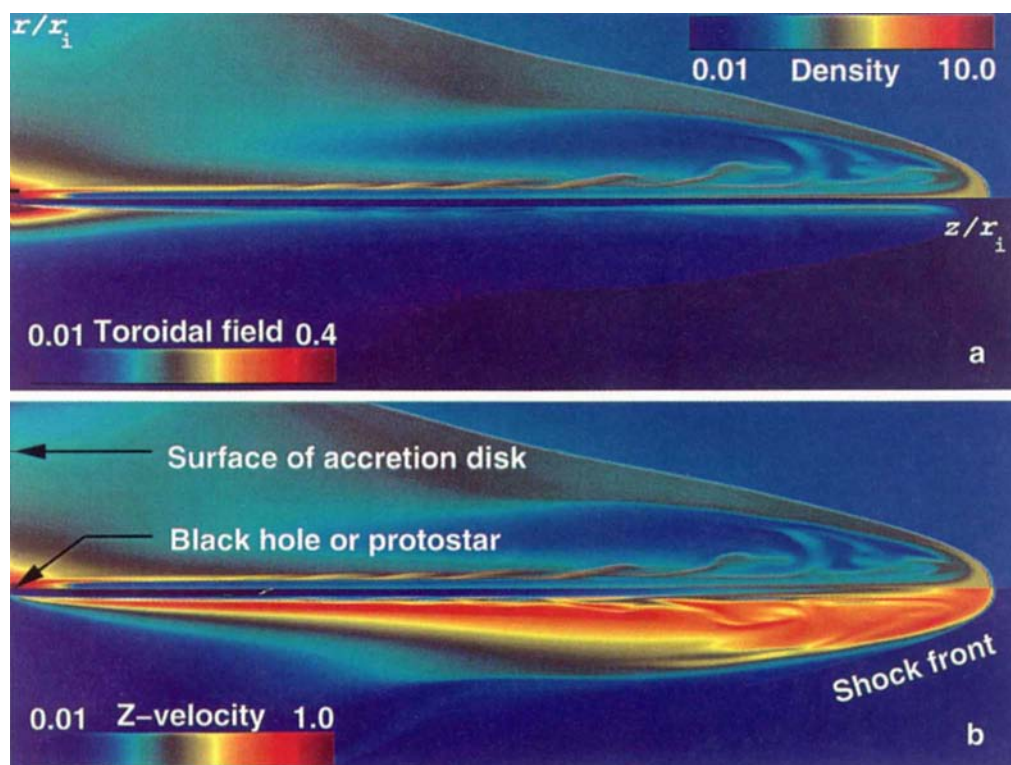


Figure 2 Illustration of the structure and dynamics of the outflow for the potential field configuration. **a** and **b**, snapshots of the flow at 180 inner time units. In **a**, the density (upper panel) is compared to the toroidal magnetic field (lower panel). The linear scale (colour palette for all figures) has red as high and blue as low value for all physical quantities. The density is given in units of ρ_i and the velocity in

units of $v_{K,i}$ in all colour plots. A tick mark on the r -axis of the upper panel marks a distance of $1 r_i$. **b** shows the evolution of the density as compared to the velocity v_z . Note that in all figures in this Article there is no outflow on radial scales $< r_i$. This is a consequence of our boundary condition that assumes the absence of a disk inside r_i .

magnetic field and vertical outflow speed as compared with the density, at time 180. An obvious bow shock traces the working surface of the outflow with respect to the undisturbed corona. There is also a highly collimated jet-like stream of gas that starts at the disk surface. The comparison of the jet density (upper panel) and toroidal magnetic field (lower panel) in Fig. 2a shows that the outflow density is delimited by the region of strong toroidal field. The physical reason for this is the strong radial pinch force that is exerted towards the jet axis by the magnetic force arising through the combination of a dominant toroidal field, and its associated current which flows primarily along the outflow axis. The velocity field (Fig. 2b) is interesting in that the highest-speed gas is nearest the outflow axis with lower speeds at larger radii.

To show the details of the acceleration mechanism, we plot in Fig. 3 the values of various physical quantities at increasing distance along a fiducial field line, anchored near $r_0 = r_i$ (r_0 is the footpoint of the magnetic field line on the disk surface). Figure 3a shows that MHD analogues of the Mach number in hydrodynamics, the Alfvén and FM Mach numbers, achieve values of 5 and 1.5 respectively. The ratio of the toroidal to poloidal field strength builds from an initial value of unity to 3 (Fig. 3b). Thus, beyond the FM surface, the toroidal magnetic field dominates the dynamics of the jet. Figure 3c shows that the poloidal speed of the gas along a field starts at its input value of one-thousandth of the Kepler speed at the base of that field line ($v_{K,0}$), reaches 1.5 times this value at the FM point, and continues to accelerate to 2.1 $v_{K,0}$ at the end of the grid. Thus, flow speed from our fiducial, low-mass young stellar object reaches upwards of 220 km s^{-1} , comparable to observed values. The density of the flow decreases as one moves outwards along the field line because of the divergence of the flow streamlines (Fig. 1c).

The outflow achieved in this simulation has properties predicted by steady MHD disk-wind theory. The acceleration of the flow from the disk occurs by a centrifugal effect whereby, at some point along sufficiently inclined field lines, centrifugal force dominates gravity and gas is flung away like ‘beads on a wire’³⁶. This toroidal field component is created because the field lines are forced to co-rotate with the underlying disk. At large z , the inertia of matter in the flow region ultimately forces the field to fall behind the rotation of the disk, producing the toroidal field component. The acceleration of material occurs in two separate stages. In stage 1 (the region along a field line between the disk and the FM point) the centrifugal effect dominates. In stage 2, (region beyond the FM point), the flow speed scales as $v_z \propto z$, which is theoretically expected when outflow becomes super-Alfvénic (supersonic for hydrodynamic jet models³⁷).

Table 1 lists the mass, momentum and energy flux rates across the outer axial boundary ($z = 80.0$) of our simulation, evaluated at 400 time units. These numbers are comparable to those for AGN and protostellar outflows observed on much larger physical scales^{38,39}. The disk accretion rate deduced from our mass outflow rate $\dot{M}_w$ is $\dot{M}_a \approx 6\dot{M}_w$.

Episodic outflow

We show a series of snapshots of the evolution of an episodic flow in Figs 4–6. The field lines comprise an initially uniform and vertical magnetic-field structure that penetrates the disk and overlying corona, perhaps produced by dragging an external magnetic field into the disk by the accretion flow^{40,41}. According to steady-state models, this configuration is unfavourable for launching an outflow. In two snapshots taken at 100 and 400 time units, Fig. 4 shows

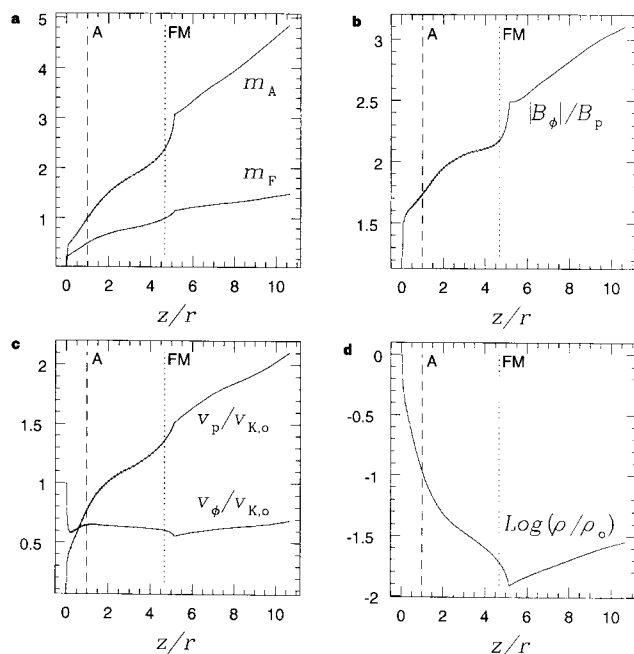


Figure 3 Physical quantities, along the field line whose footpoint is anchored at r_i , plotted against $z/r = \cotan^{-1}(\psi)$ where ψ is the polar angle as measured from the origin of the coordinate system. **a**, Alfvén Mach numbers m_A and the fast magnetosonic (FM) numbers m_F ; **b**, the ratio of the toroidal to poloidal magnetic field along the field line; **c**, the poloidal velocity v_p and the toroidal velocity v_ϕ , in units of the Kepler speed at the footpoint of the field line; the density ρ in units of the density at the footpoint of the field line. The position of the Alfvén critical point (marked A) and the FM point (marked FM) on the field line are also given. This figure can be compared with the results in Fig. 4 of ref. 17.

that outflow is nevertheless launched from the accretion disk for reasons we describe below. The highly collimated, jet-like flow has a density structure that is dominated by discrete knots. At time 100 the working surface of the outflow is clearly evident. The working surface in this simulation has the shape of a cone that advances into the flow with a swept-back bow shock along its flanks^{42,43}. This surface leaves our grid long before 400 time units have elapsed.

The density structure in this simulation never achieves steady state. We have run simulations for up to 1,000 time units and find that the episodic nature of the flow persists unabated. The density contours show that the jet is strongly confined near the axis. We find that the associated toroidal field in the flow is tightly anti-correlated with the knots⁴³; high (low) density regions are associated with the lowest (highest) toroidal field strength. The average speed of material in this outflow is $0.5v_{K,i}$, with the highest velocities $\sim v_{K,i}$. Although this velocity is not as large as is sometimes observed on larger scales in real outflows⁴⁴, our simulations explore only a limited region of physical and parameter space. A dynamic view of this simulation (available at http://www.physics.mcmaster.ca/Grad_Ouyed/ROuyed.html) shows that the knot generator stays fixed in space and that the knots, once formed, persist as coherent structures that propagate down the length of the jet. The large knots in our simulation appear to be more slowly moving structures that build up by absorbing the more quickly moving knots produced at the generator.

To explore the knot-generating region further, we performed a simulation at four times the axial and twice the radial resolution, but otherwise identical to that shown in Fig. 4; $(z \times r) = (20 \times 10)r_i$. In Fig. 5 we show two snapshots of this innermost region of the outflow, at times 37.2 (Fig. 5a) and 48.0 (Fig. 5b). The upper panel in each of these snapshots shows the flow density, and the lower panel, the associated flow velocity v_z . In Fig. 5 we see a knot (labelled A), and 11 time units later, in Fig. 5b we see a newly formed knot (B) in the same position as A had, while knot A has

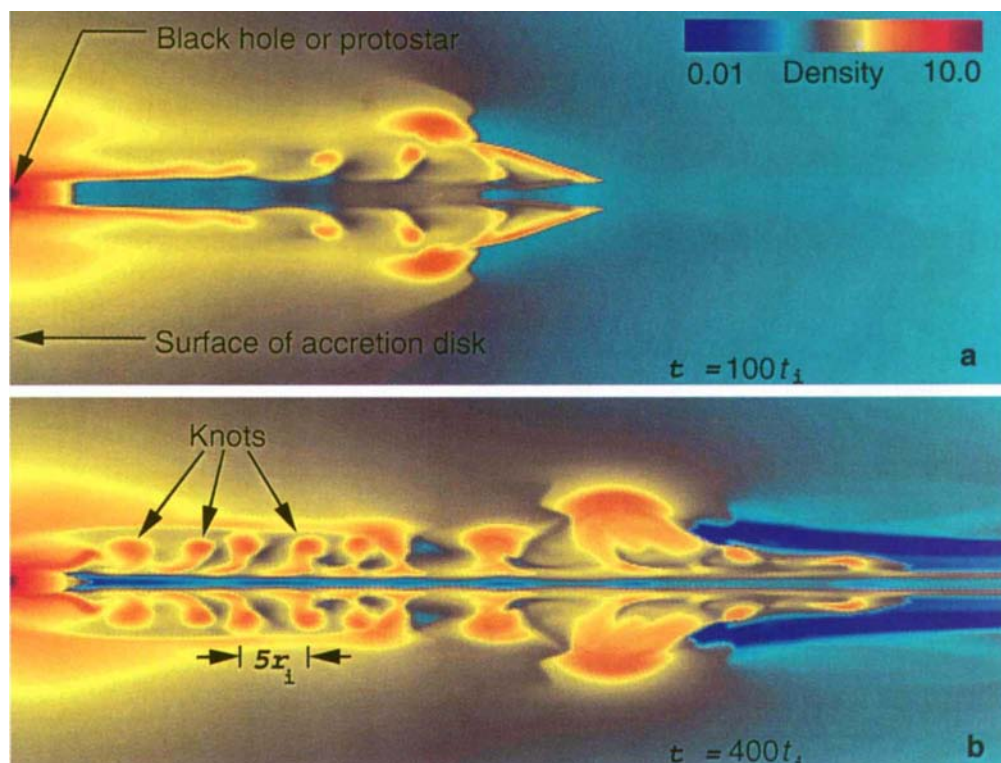


Figure 4 Snapshots of the jet density at 100 (**a**) and 400 (**b**) inner time units. The jet is produced in an initially uniform and vertical magnetic field described by the vector potential $A_\phi \propto r$.

Table 1 Jet units and energetics

	Proto-star	Black hole
Mass ($M_\odot$)	0.5	10^8
r_i (AU)	0.04	20.65
$v_{K,i}$ (km s $^{-1}$)	120.0	6.7×10^4
t_i (d)	0.62	0.53
$\dot{M}_w$ ($M_\odot$ yr $^{-1}$)	9.1×10^{-7}	2.2×10^{-2}
$\dot{M}_w v$ ($M_\odot$ yr $^{-1}$ km s $^{-1}$)	2.8×10^{-5}	7.5×10^2
$\frac{1}{2}\dot{M}_w v^2$ (erg s $^{-1}$)	8.0×10^{32}	1.2×10^{43}

Here r_i is the innermost radius of the accretion disk (r_i could be defined as being the surface of the star, the magnetopause radius of a magnetized star and surrounding disk; or the last stable keplerian-like orbit around a black hole for active galactic nuclei); $v_{K,i} = \sqrt{GM/r_i}$ is the Kepler speed at r_i and $t_i = r_i/v_{K,i}$ is the time unit. $\dot{M}_w$, $\dot{M}_w v$ and $\frac{1}{2}\dot{M}_w v^2$ are the mass, momentum and energy flux rates across the outer axial boundary ($z = 80.0$) of our simulation, evaluated at 400 time units.

moved down the flow. Both knots are produced at a distance of $z_{\text{knot}} \leq 6 - 7r_i$ from the central source.

In Fig. 6 we show the magnetic field configuration of the outflow at times 37.2 (Fig. 6a), 42.6 (Fig. 6b) and 48.0 (Fig. 6c) during which the new knot (B) is formed (knot A moves away from the generating region as knot B is formed). This process takes a time $\tau_{\text{knot}} \approx 11$ time units. Comparison of Fig. 6a and c shows that knots are spatially separated by a distance of $\delta z_{\text{knot}} \approx 5r_i$ and thus move at a speed of $\sim 0.5v_{K,i}$ out of this generating region.

How is the outflow and its knots generated? The plots in the right-hand panels of Fig. 6a–c reveal that a narrow radial region of field lines in the innermost parts of the disk has been opened up, making an angle of 50° with respect to the disk surface. This narrow region drives the outflow and is produced by the toroidal field which is strongest in the inner regions of the outflow where the underlying Kepler rotation is the largest. A radial, outwardly directed, toroidal magnetic pressure gradient is produced by the newly created toroidal field and this pushes open the field lines setting up a condition favourable for outflow. The knots are produced in a region of the flow beyond the Alfvén surface (marked by large dots in Fig. 6c) and the FM surface in the outflow.

The size of the wind-production region on the surface of the disk depends upon the ease with which field lines can be pushed aside by the toroidal field pressure. The rigidity of the field is measured by the β parameter and is very different for our two cases. For the more magnetically dominated episodic case, the magnetic pressure associated with the self-generated toroidal field in the narrow band of outflowing gas has a local maximum so that the pressure force acts both radially outwards from, and inwards towards, the axis. The accelerating outflow, on encountering the inwardly directed pressure gradient, is reflected back towards the axis. But because the outflow is rotating, the gas spins-up as it moves inwards and reflects off an inner ‘centrifugal barrier’ when it reaches a radius comparable to its footpoint radius^{20,45,46}, as Fig. 6 clearly shows (the field lines in Fig. 6a and c show that the flow recollimates towards the axis, and then reflects back into the slower-moving body of the jet). The resulting nearly harmonic oscillation in the width of the flow²⁴ between these two ‘barriers’ must, by mass conservation, lead to variations in flow velocity (much like a constricted garden hose). These variations in flow speed rapidly steepen into fast MHD shocks⁴⁶. Estimating that the toroidal Alfvén speed in our simulation is $v_{A,\phi} \approx (0.5-0.6)v_{K,i}$, and the width of the jet to be $\delta r_j \approx (3-4)r_i$, the oscillation period of the jet is $t_{\text{osc}} \approx 2\delta r_j/v_{A,\phi} \approx (11-13)t_i$, which is the knot production timescale. By contrast, the initial magnetic pressure in the steady flow case drops significantly as one moves outwards for the axis. The accelerating outflow does not encounter a strongly magnetically overpressured outer barrier, and continues to expand radially finally achieving a quiet, cylindrically collimated state.

Outflow formation may be a robust process wherein the keplerian disk generates the toroidal magnetic field whose radial pressure gradient sculpts a favourable coronal magnetic field structure. Jets

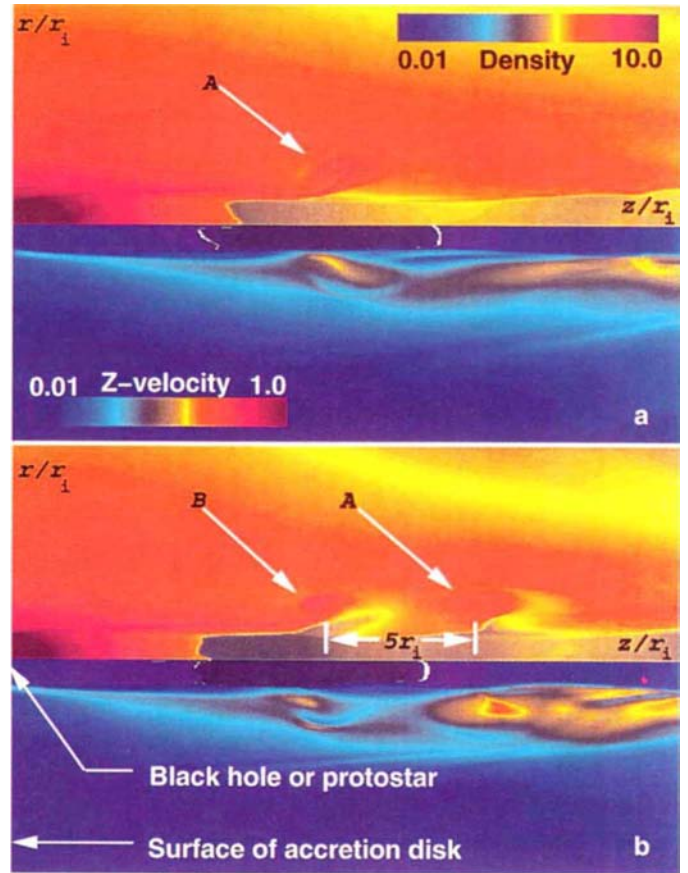


Figure 5 Close-up snapshots of the knot generating region of the outflow at 37.2 (a) and 48.0 (b) inner time units. In a and b, the density is in the upper panel (the tick mark on the r -axis denotes a distance of $1r_i$), and the v_z i the lower panel. Two knots, denoted A and B, are shown which develop within a background of the faster-moving flow. These knots are separated by a region of high toroidal magnetic-field strength.

are capable of producing episodic events⁴⁷; our simulations show that fast knot production timescales occur which are a factor of a few shorter than is observed for knot emergence in jets on much larger scales. Finally, gas in our episodic model has significant rotation $\sim 0.6v_{K,i}$ on the innermost scales which agrees with observations of rotating, outflowing gas within $12r_i$ of certain young stellar objects⁴⁸.

Transition from episodic to stationary flow

Given the importance of toroidal field in producing episodic jet behaviour, what happens if the ram pressure of gas injected into the corona is comparable to the confining pressure of the toroidal field? The preceding discussion suggests that when the number $N = (B_\phi^2/8\pi v^2)_{\text{inj}} > 1$ (which is a constant for the uniform field configuration), then the outer, toroidal barrier is strong and flow should be episodic (in Figs 4–6, $N = 36$). But if $N < 1$, then the toroidal magnetic pressure should be overwhelmed and a stationary flow should result. We performed a series of eight simulations identical to those shown in Figs 5 and 6 except that the injection speed in the initially uniform vertical field configuration was varied, from a minimum value of 0 to a maximum of $10^{-2}v_{K,i}$. For injection speeds 5 and 10 times larger than those shown in Figs 1–6 (for which $N = 1.45$ and 0.36 , respectively), stationary outflows did indeed develop whose terminal speeds were $\sim v_{K,i}$. For injection speeds 5 and 10 times smaller (for which $N > 1$), the outflows were episodic and had terminal speeds and knot properties similar to

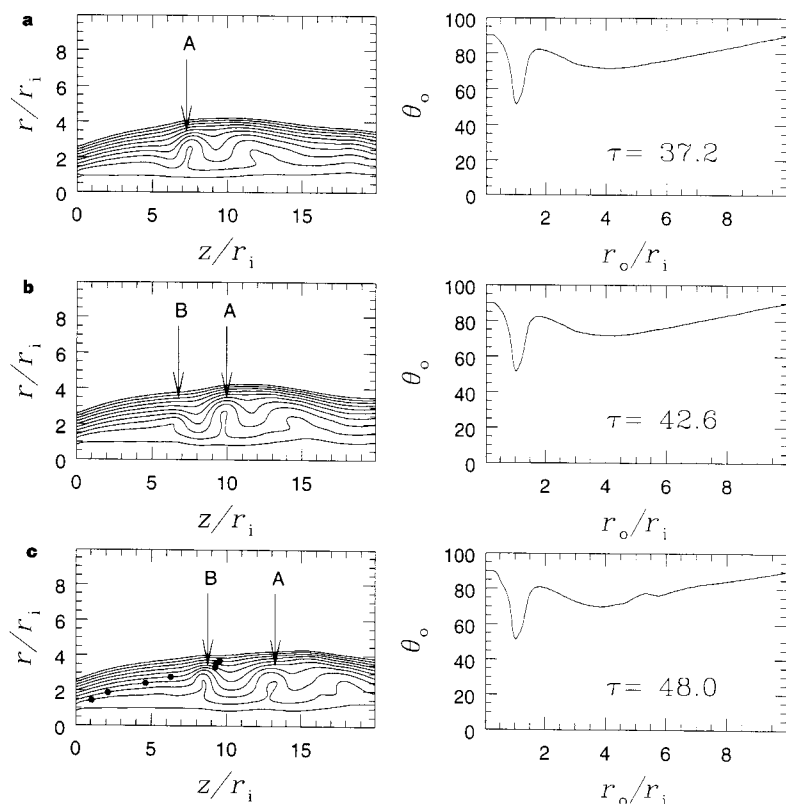


Figure 6 The left panels show the magnetic-field structure of the knot-generating region at three times; 37.2 (a), 42.6 (b) and 48.0 (c) inner time units. The right panels show the angle θ_o of field lines at the base of the flow, with the disk surface, at these times. Note the narrow band of field lines which is sufficiently opened

($\theta_o \approx 60^\circ$) so as to drive the outflow. Only field lines involved in the knot-generation process are shown; field lines at larger disk radius stay reasonably vertical, as seen in the right panels.

those reported above. In the simulation where there was no mass injection, a transient outflow composed of coronal gas developed which gradually died out as the initial coronal material in the jet zone was depleted. Experiments with our potential flow configuration showed that at mass injection speeds of $v_{\text{inji}} = 10^{-4} v_{\text{K,i}}$, rather diffuse knots appeared but these appeared to dissipate as they moved away from the generating region.

Our simulations only sparsely sample a rather large parameter space, and have much smaller scales than can be directly imaged by the Hubble Space Telescope. The major constraint in our study is that the accretion disk is not allowed to respond to the changing torques exerted by the jet. In spite of the limitations, we suggest that these simulations provide some important insights into the nature of astrophysical jets. $\square$

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Large-scale magnetic fields in the outflow from the young stellar object T Tauri S

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T Tauri stars are young stellar objects, similar in mass to the Sun, that are completing the transition between a collapsing cloud and a main-sequence star powered by hydrogen fusion¹. Many of these objects are associated with circumstellar (and, presumably, protoplanetary) disks², as well as energetic outflows of gas that can extend several light years away from the young star³. These outflows are thought to be collimated by magnetic fields⁴, but direct observational evidence for such fields has hitherto been lacking. Here we show that the infrared companion⁵ (T Tau S) of the prototypical T Tauri star (T Tau itself) recently ejected in opposite directions two large lobes of mildly relativistic particles. The radio emission from the two lobes exhibits strong circular polarization of opposite helicity, implying the existence of a strong, ordered magnetic field at a surprisingly large distance from the source. We also find that the T Tau system may contain three stars, rather than two as previously thought.

Since the discovery that T Tauri stars are progenitors to stars like our own Sun, they, and their prototype T Tau, have been objects of intense study¹. T Tau itself is associated not only with a well-known reflection nebula (Hind's Nebula or NGC1555) but is located at the centre of a diffuse Herbig–Haro-type emission region (Burnham's Nebula) powered by shocks^{6,7}. The optically unseen companion to T Tau, T Tau S, has been shown using infrared speckle interferometry to lie ~ 0.7 arcsec south⁸ of the visible star (see also Fig. 1). Proper-motion studies⁹ have implied that the optical star and T Tau S almost certainly form a gravitationally bound system, the projected separation of which is decreasing at a rate of ~ 8 mas yr⁻¹. Both T Tau N, as the optical star is known, and T Tau S are radio sources^{10,11} and the centimetre emission from T Tau S is known to be variable on timescales of a few days and to be circularly polarized^{11,12}. The presence of a third star (some four magnitudes dimmer than T Tau N in the optical) was claimed^{13,14} on the basis of speckle imaging observations but its existence has been subsequently questioned¹⁵. Examination by us of a Hubble Space Telescope archive image taken in the V band confirms the lack of an optical companion.

On 29 and 30 November 1992, we used the Multi-Element Radio Linked Interferometer Network (MERLIN) based at Jodrell Bank to map T Tau at 6 cm wavelength in phase referencing mode. The bandwidth of the observations was 15 MHz and all four Stokes parameters were recorded. The total observing time on source was 10.6 h. Calibration and mapping were carried out using the Astronomical Image Processing Software (AIPS) package. 3C286 was used as our flux and polarization calibrator and we assumed a total flux density for this source of 7.382 Jy at 4,993 MHz. The beam size was 0.162×0.153 arcsec² but was smoothed to a diameter of 0.2 arcsec to improve the signal-to-noise ratio. The resultant r.m.s.

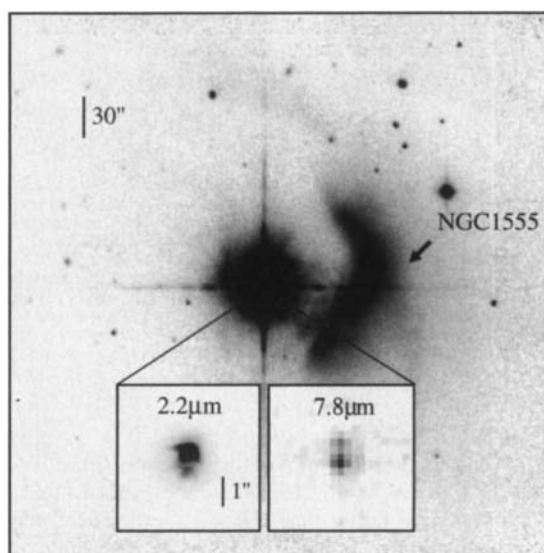


Figure 1 T Tau (centre) as seen in the near-infrared (K band). Hind's Nebula (NGC1555) is to the right. North is up and east is to the left. T Tau S, the infrared companion, is ~ 0.7 arcsec south of the optical star and is therefore not seen in the wide-field image. It can, however, be observed in the inset 2.2- μ m and 7.8- μ m images from the Calar Alto and UKIRT Observatories respectively.

noise level was 170 μ Jy per beam and the final field of view was 7.68×7.68 arcsec².

Figure 2 shows our 6-cm radio image of T Tau. Apart from the two well-known radio sources (T Tau N and T Tau S), it is immediately seen that there is extended emission between the two stars, as well as a third source which we shall refer to as T Tau R. An examination of the original Very Large Array (VLA) discovery image¹⁰ of T Tau N and T Tau S in fact shows extended radio emission north of T Tau S with two apparent local maxima. If we identify T Tau R with the more southern of these two maxima and T Tau N with the other, then it would appear that T Tau R was originally¹⁰ (and incorrectly) identified with the optical star. However, subsequent observers^{11,16} used the name T Tau N for the radio source that really is coincident with the optical star T Tau (see Table 1). We shall stick to the nomenclature in current use and as illustrated in Fig. 2. We mention in passing, however, that this finding explains why the separation between what was originally called 'T Tau N' and T Tau S apparently 'increased' between June 1982¹⁰ and April 1990¹¹ from 0.54 to 0.62 arcsec respectively, whereas, according to the relative motion of these two sources measured using infrared speckle imaging, we would have expected their angular separation to have decreased⁹.

Table 1 lists the observed positions of the radio sources as well as that of the optical star (also marked in Fig. 2) using positional and proper-motion data derived from the Carlsberg Automatic Meridian Circle (CAMC) transit telescope¹⁷. The separation of T Tau N and T Tau S was measured to be 0.63 ± 0.03 arcsec (epoch J1992.9) at a position angle (PA) of $180^\circ \pm 1^\circ$ (here PA is measured in the direction north through east centred on T Tau N). Assuming the identifications mentioned above for the original VLA discovery image, we can compare these values with those for June 1982¹⁰ when the separation between T Tau N and T Tau S was 0.71 ± 0.03 arcsec and the PA was $172.5^\circ \pm 1^\circ$. The observed changes are not only in excellent agreement with the expected relative motion in both right ascension and declination based on the speckle data⁹ but also clearly identify the radio source T Tau N with the optical star, as does the revised optical position. Moreover T Tau R has also apparently changed position over the same timescale with respect to T Tau N: while their separation has remained virtually constant at

Table 1 Radio positions and flux densities for sources in the T Tau region

Source	RA (B1950) (h min s)	Dec. (B1950) (° ' ")	I (mJy)	R (mJy)	L (mJy)
T Tau N	04 19 04.246	+19 25 05.17	0.9	0.5	1.3
T Tau R	04 19 04.243	+19 25 04.88	0.8	0.6	1.1
T Tau S	04 19 04.246	+19 25 04.54	2.1	2.4	1.8
T Tau (optical)	04 19 04.240	+19 25 05.26	–	–	–

All positions quoted are equinox B1950.0 and epoch J1992.9 and are determined from the total intensity radio map. The optical position for T Tau is also given for reference and was determined by the Carlsberg Automatic Meridian Circle (equinox B1950.0, epoch J1986.9) as 04 h 19 min 04.233 s, +19° 25' 05.34" with a proper motion of $+0.0011 \pm +0.0003 \text{ s yr}^{-1}$ and $-0.013 \pm 0.003 \text{ arcsec yr}^{-1}$ in RA and dec. respectively. The standard errors in the optical position are $\pm 0.1 \text{ arcsec}$ whereas those in the radio are approximately $\pm 0.035 \text{ arcsec}$. The flux densities are given for total intensity (I) as well as right (R) and left (L) circular polarization components; errors in these quantities are $\pm 0.15 \text{ mJy}$. Peak flux densities are given except in the case of the extended emission from T Tau S where the total flux density lying within the 2.5σ contour is quoted.

$0.29 \pm 0.03 \text{ arcsec}$, the relative PA of these two sources has decreased from $230^\circ \pm 3^\circ$ to $195^\circ \pm 3^\circ$. This would imply that T Tau R is part of the T Tau system rather than a background source, the much smaller proper motion of which would be clearly visible over this period. Its inferred low velocity relative to T Tau N ($\sim 10 \text{ km s}^{-1}$, assuming the Taurus Auriga cloud is at a distance of 140 pc; ref. 18) almost certainly precludes T Tau R from being a Herbig–Haro object. This suggests that T Tau R may be a highly embedded star, as it has not been seen using speckle interferometry, possibly in orbit around T Tau N.

Turning now to T Tau S, the brightest radio source in the system, we mentioned previously that this star is known to be occasionally circularly polarized^{11,12}. We therefore examined both left and right circularly polarized channels of our radio image, and found that the radio emission from T Tau S divided into two spatially separated lobes (Fig. 3) of opposite helicity. The centroids of emission from the two lobes were $\sim 0.15 \text{ arcsec}$ apart (corresponding to a projected separation of 20 AU). This is not only the first time that extended polarization has been observed at radio wavelengths from a pre-main-sequence star but is also the first unambiguous evidence for the presence of large-scale magnetic fields (that is, fields extending for tens of AU rather than several stellar radii) surrounding such stars. Here, the only viable means^{11,19} for generating the observed circularly polarized emission appears to be gyrosynchrotron radiation from mildly relativistic (Lorentz factor $\gamma \approx 2\text{--}3$) electrons. The gyrofrequency ν_B is given by²⁰

$$\nu_B = 2.8 \times 10^6 B \text{ Hz} \quad (1)$$

where B is in gauss, and the fractional circular polarization π_c by²⁰

$$\pi_c \approx 1.26 \times 10^{0.035\delta} 10^{-0.071\cos\theta} \left(\frac{\nu}{\nu_B} \right)^{-0.782+0.545\cos\theta} \quad (2)$$

Here δ is the power-law index for the electron energy distribution (that is, $n(E) = KE^{-\delta}$, where K is a constant, E is the energy, and we have assumed an isotropic pitch angle distribution), θ is the angle between the magnetic field direction and the line of sight, and ν is the observing frequency. Given the high degree (up to $\sim 30\text{--}40\%$) of circular polarization found in each lobe at $\nu = 5 \text{ GHz}$, it is clear from equations (1) and (2) that even for such an extreme value of δ as 4 and $\theta \geq 20^\circ$, the field strengths must be at least several gauss in the emitting regions. Extrapolating these values back to the star itself, it is immediately seen that for a dipolar field (that is, $B \propto r^{-3}$), or an even slower r^{-2} decrease, where r is the radial distance, the inferred magnetic fields close to the star are unrealistically high. What evidence there is suggests that the magnetic field strengths near the stellar surface are at most several kilogauss at least for weak-line T Tauri stars²¹. Thus we are forced to conclude that the fields giving rise to the circularly polarized emission are part of a

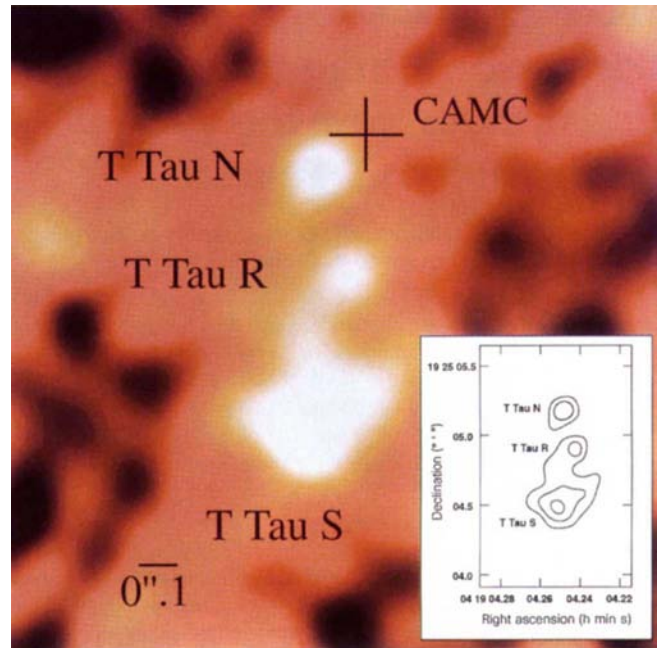


Figure 2 MERLIN 6-cm intensity image of T Tau. In addition to the known radio sources T Tau N and T Tau S, extended radio emission is observed between these two stars. There is also a third source which may have been wrongly classified as the optical star in the original Very Large Array discovery image (see text). The radio emission from T Tau S appears extended in the northwest–southeast direction due to an outflow from this highly embedded young star. The position of the optical star, as derived from the Carlsberg Automatic Meridian Circle (CAMC), and allowing for proper motion, is also shown. The cross indicates the 1σ errors in the optical position. Inset, contour plot of the same data of the T Tau region (B1950 coordinates) contours are at 0.4, 0.7 and 1.2 mJy.

collimated outflow from T Tau S. Given the strengths of these fields so far from their source, we speculate that they may have been amplified by way of compression in radiative shocks within the outflow. Such shocks may also be responsible for accelerating the mildly relativistic electrons that give rise to the gyrosynchrotron emission.

The projected direction of the outflow from T Tau S, based on our radio data, is northwest–southeast, roughly coincident with the axes of the larger-scale optical and infrared outflows that are thought to arise from this star^{22,23}. Moreover, it appears that T Tau S underwent a major outburst in the near-infrared, probably of the EX Lupi type²⁴, starting in 1990⁸. Such temporary increases in luminosity are almost certainly due to a rise in the accretion rate of the T Tauri star and, as accretion and outflow rates are related³, one would expect a corresponding change in the outflow. It is thus interesting to note that whereas the radio emission from T Tau S appears point-like in earlier maps¹¹ it is extended in our MERLIN data taken some two years after the outburst. Assuming the left- and right-circularly polarized lobes are $\sim 0.1 \text{ arcsec}$ in width, then given their flux densities we can derive a remarkably high brightness temperature for the emission of $\sim 2 \times 10^5 \text{ K}$. Finally, we remark that the radio emission from T Tau N is also seen (Fig. 3) for the first time to be circularly polarized. As previous observers¹¹ failed to detect this, we must infer that its polarization, as in the case of T Tau S, is variable.

Our observation that strong, and what are almost certainly dynamically important, magnetic fields are present in the extended outflow from a young star suggests that such fields play an important role in their collimation. Whereas virtually all of the other fundamental outflow parameters (such as mass loss rates and

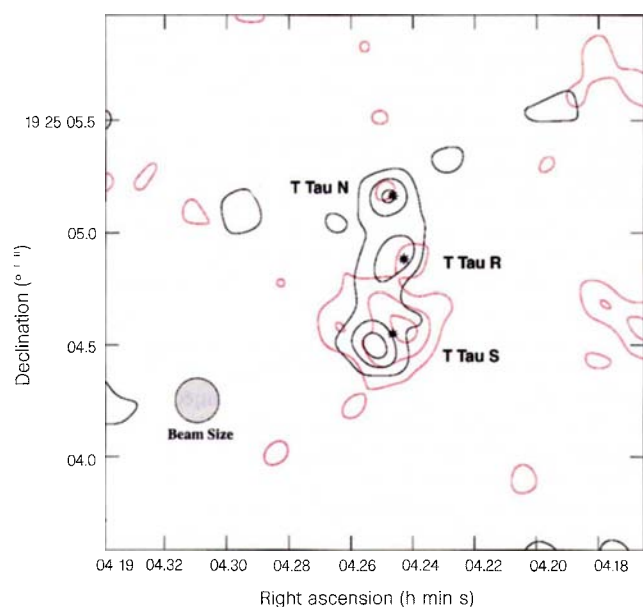


Figure 3 MERLIN contour map of the 6-cm radio emission in the T Tau region. Right- and left-hand circularly polarized components are shown in red and black respectively. Contour levels are in each case 0.4, 0.8 and 1.2 mJy, right ascension and declination are in B1950 coordinates. Note the spatially resolved separation of the emission from T Tau S into two lobes of opposite helicity and the net left-hand circular polarization of T Tau N. Thus the 'flow' and 'counterflow' from T Tau S are both circularly polarized but in opposite senses. The source positions (see Table 1) are shown as asterisks.

velocities) have been determined through imaging and spectroscopic studies³, magnetic field strengths have remained elusive. Polarization studies at a range of radio frequencies could provide us with the necessary diagnostic tool. Moreover, given that the radio emission from a number of other outflows is suspected of being non-thermal²⁵, such studies could in principle allow us to test currently favoured models of the magnetic collimation of outflows from young stars. We intend to continue monitoring T Tau S outflow at radio wavelengths to see how this outflow evolves with time. It should be possible not only to test the shock hypothesis but also, through proper-motion studies, to link unambiguously the radio outflow activity with what was probably a major accretion event in the life of a newborn star. □

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Phase measurement in a quantum dot via a double-slit interference experiment

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The transport properties of electronic devices are usually characterized on the basis of conductance measurements. Such measurements are adequate for devices in which transport occurs incoherently, but for very small devices—such as quantum dots^{1,2}—the wave nature of the electrons plays an important role³. Because the phase of an electron's wavefunction changes as it passes through such a device, phase measurements are required to characterize the transport properties fully. Here we report the results of a double-slit interference experiment which permits the measurement of the phase-shift of an electron traversing a quantum dot. This is accomplished by inserting the quantum dot into one arm of an interferometer, thereby introducing a measurable phase shift between the arms. We find that the phase evolution within a resonance of the quantum dot can be accounted for qualitatively by a model that ignores the interactions between the electrons within the dot. Although these electrons must interact strongly, such interactions apparently have no observable effect on the phase. On the other hand, we also find that the phase behaviour is identical for all resonances, and that there is a sharp jump of the phase between successive resonance peaks. Adequate explanation of these features may require a model that includes interactions between electrons.

In a previous interference experiment³, which exploited an Aharonov–Bohm (AB) ring (see ref. 4 for review) and a quantum dot (QD) imbedded in it, the conductance was shown to depend not only on the magnitude of the transmission through the QD, but also on the phase acquired by electrons traversing the QD. The observed oscillatory behaviour of the conductance, G , which was periodic with the flux quantum $\Phi_0 = h/e$ (h is Planck's constant, e is the charge on the electron), confirmed that the QD supports coherent transport. As the experiment was set up with a two-terminal configuration, however, current conservation and time reversal symmetry lead⁵ to $G(B) = G(-B)$ with B the magnetic field, restricting the phase of the AB oscillations to be either 0 or π . This means that only abrupt jumps between the two allowed phase values are possible^{6,7}. Physically, this is a direct result of interference between the multiple paths that traverse the ring, obscuring the phase evolution of the transmission coefficient of the QD^{7–10}. This phase rigidity does not exist in a double-slit-like interference experiment with a four-terminal configuration^{5,11}. We used such a

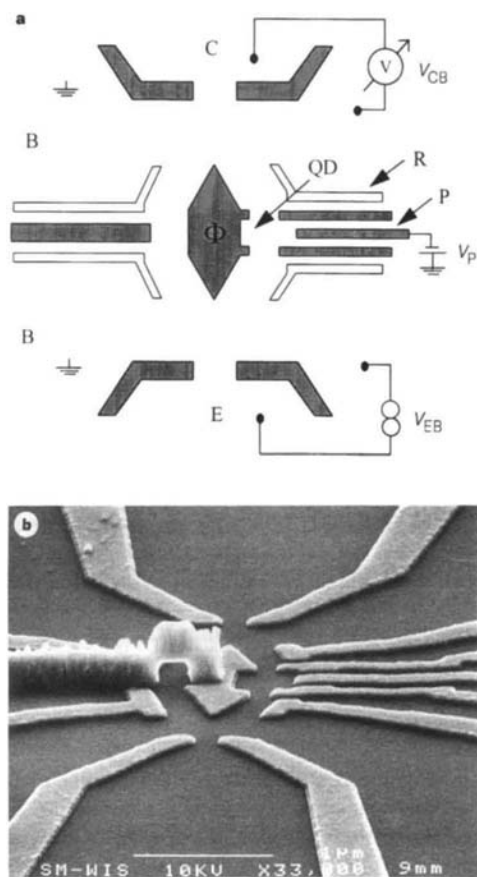


Figure 1 **a**, Schematic description of the double-slit interference experiment with a QD replacing one slit. The system is composed of three different regions, an emitter E, a collector C, and a base region B on both sides of the two slits. A contact to each region allows a four-terminal measurement. Reflector gates (R) are drawn in white. The excitation voltage V_{EB} is applied between emitter and base. The collector voltage V_{CB} is measured between base and collector. A voltage V_P on the plunger gate P changes the occupation of the QD. **b**, A top-view scanning electron micrograph of the device. The grey areas are metallic gates deposited on the surface of the heterostructure. The negatively biased gates define emitter and collector QPCs, a slit and a QD. A special lithographic technique, involving a metallic air bridge, is used to contact the central gate (which depletes the area between the two slits). The QD has an area $0.4 \times 0.4 \mu\text{m}^2$ with both of its QPCs and the plunger gate (P) individually controlled. Reflector gates are added to increase the measured signal.

configuration to measure directly the magnitude and the phase of the transmission coefficient through a QD in the Coulomb blockade (CB) regime.

The actual double-slit interferometer consists of a patterned, high-mobility, two-dimensional electron gas, electron density $n_s = 3.0 \times 10^{11} \text{ cm}^{-2}$, mobility $\mu = 1.6 \times 10^6 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ at temperature $T = 4.2 \text{ K}$ formed 60 nm below the surface of a GaAs–AlGaAs heterostructure, with an elastic mean free path $l = 15 \mu\text{m}$. The potential barriers are defined by negatively biased metal gates deposited on the surface of the heterostructure. Our four-terminal configuration (see Fig. 1a) consists of emitter E and collector C constrictions, called quantum point contacts (QPC), and a base region B in between. The base contacts serve as draining reservoirs with a chemical potential $\mu_B = 0$. The E and C constrictions are separated by a barrier with two openings (slits); one slit consists of the QD whose behaviour we want to measure, and the other is a reference slit in a form of another QPC. The number of electrons in the QD is around $N_{el} \approx 200$, with an average energy spacing

between discrete states $\Delta E \approx E_F/N_{el} = 55 \mu\text{eV}$, where E_F is the Fermi energy. Collector and emitter QPCs support only one transverse mode, thus producing a planar electronic wave front in the far field. As the collector signal is too small to be measured in an entirely open configuration, we incorporate additional barriers which reflect the emitted diverging electrons into the two slits and subsequently towards the collector (the white gates in Fig. 1a, called R). All measurements are done in a dilution refrigerator ($T_{\text{lattice}} = 15 \text{ mK}$, $T_{el} \approx 80 \text{ mK}$) with an a.c. excitation voltage $V_{EB} = 10 \mu\text{V}$ applied across the emitter QPC.

At low temperatures both the phase coherence length and the elastic mean free path of the electrons exceed the entire sample size. Using the multiprobe conductance formula⁵, we can see that the current at the collector QPC, I_C , is given by $I_C = (2e^2/h)(\tau_{EC}V_{EB} \pm \tau_CV_{CB})$, where V_{EB} is the injection voltage, and τ_{EC} and τ_C are the transmission probabilities from emitter to collector and through the collector QPC respectively. The open-circuit collector voltage ($I_C = 0$), $V_{CB} = (V_{EB}/\tau_C)\tau_{EC}$, leads directly to the transmission probability τ_{EC} , which in turn is a coherent sum over all path amplitudes from E to C. For the double slit case $t_{EC} = t_{QD} + t_{sl}$, where $|t_{EC}|^2 = \tau_{EC}$, t_{QD} is the transmission amplitude associated with the path traversing the QD, and t_{sl} refers to the reference path passing through the QPC slit on the left. A magnetic flux, Φ , threading the area, A, enclosed by these two classical paths results in an AB phase difference $\Delta\varphi = 2\pi\Phi/\Phi_0$ between the two interfering paths. For single-channel transmission:

$$\tau_{EC} = |t_{QD} + e^{i\Delta\varphi}t_{sl}|^2 \quad (1)$$

Assuming fully coherent transport through the QD, the interference term is proportional to $|t_{sl}||t_{QD}|\cos[\Delta\varphi + \theta(t_{sl}) - \theta(t_{QD})]$, where $\theta(t_{sl})$ and $\theta(t_{QD})$ account for the phase accumulated in the two corresponding paths. The transmission probability τ_{EC} is therefore expected to oscillate as a function of magnetic field with a period corresponding to the addition of one flux quantum to the area enclosed by the paths. As the phase accumulated in the reference path is to a good approximation constant, a change in the phase of t_{QD} leads to a similar change in the phase of the oscillating collector signal.

One must make sure that the added reflectors (R) are not preventing backscattered electrons from being collected by the base contacts; otherwise multiple path interference might occur, and higher-periodicity AB oscillations and phase rigidity might dominate⁷. We checked this by opening the QD, leaving only one of its QPCs functional, so that this served as an adjustable barrier adding to the accumulated phase in this path. As hoped, we found a smooth phase shift in the oscillating collector voltage with no higher harmonics (with periods Φ_0/n) as this QPC was adjusted. These results strongly suggest that the two direct paths predominantly contribute to the interference.

The QD is defined by adjusting the resistance of its QPCs to be greater than $h/2e^2$, so that it is in the CB regime with well separated energy levels, namely $\Gamma < \Delta E$, where Γ is the resonance width. Moreover, because we adjust the openings so that $k_B T < \Gamma$ ($k_B T \approx 7 \mu\text{eV}$), the width of the conductance peak is determined by Γ and not by the temperature. Scanning the plunger gate voltage, V_P , we find pronounced resonances in V_{CB} , as expected for a QD in the CB regime (Fig. 2a), on top of a large background that results from the reference path. The asymmetry of the resonance peaks is a result of interference with the reference path. When a magnetic field is applied and the QD is being tuned to conduct, the collector signal shows AB oscillations with the expected period $\Delta B = \Phi_0/A = 3.5 \text{ mT}$. This confirms the results of Yacoby *et al.*³ that transport through the QD is coherent. Figure 2b shows examples of AB oscillations measured at four specified points on the Coulomb peak seen in Fig. 2a. A smooth phase shift of the AB oscillations, on the scale of the resonance width, is observed. This phase shift gives

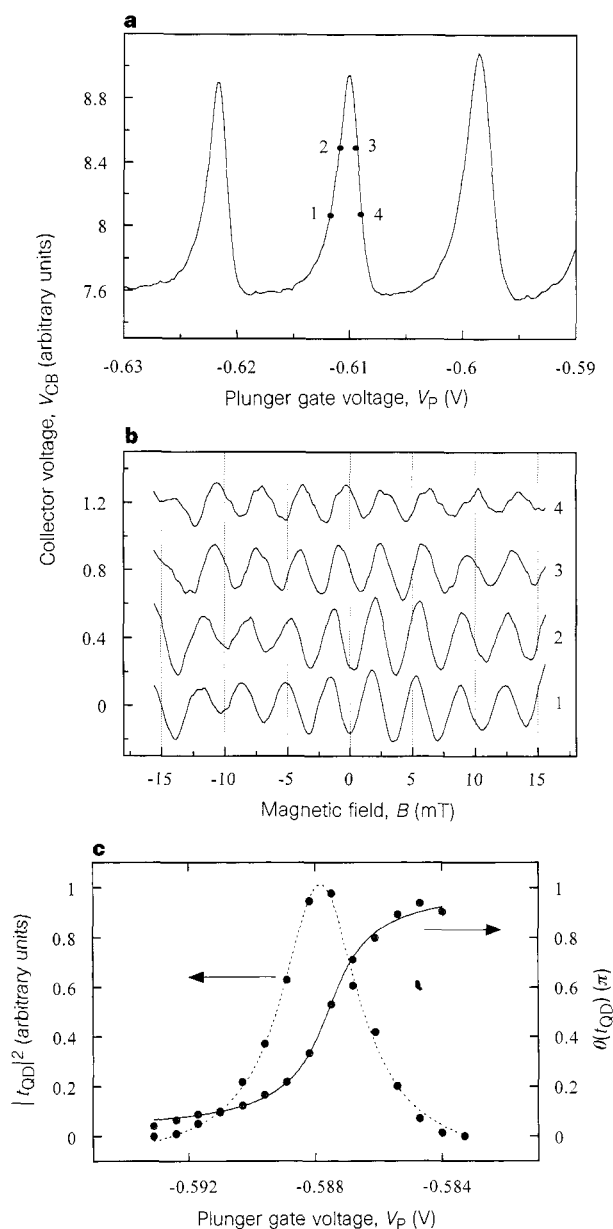


Figure 2 **a**, Resonance peaks as a function of the plunger gate voltage. **b**, A series of interference patterns taken at specified positions on a peak in **a**. The traces are shifted vertically for clarity. **c**, Bare resonance peak $|t_{\text{QD}}|^2$, obtained from the magnitude of the AB oscillations, and absolute value of the accumulated phase $\theta(t_{\text{QD}})$. The expected phase behaviour in a Breit-Wigner model (solid line) and a Lorentzian fit to the resonance peak (dotted line) are shown.

directly the phase shift of the transmission coefficient of the QD. For a complete picture we record the AB interference oscillations at many points along a resonance peak and do a complex Fourier transform of the data. The phase evolution along one peak, seen in Fig. 2c, shows a monotonic rise by almost π near the maximum of the resonance.

We model the QD as a double barrier system confining a well with quasi-bound states. The transmission amplitude through such a system shows resonances near the energies of the quasi-bound states, E_n , described by the Breit-Wigner formula¹²

$$t_{\text{QD}} = C_n \frac{i\Gamma/2}{E - E_n + i\Gamma/2} \quad (2)$$

where E is the energy of the incident carriers, Γ is the width of the

resonant level, and C_n is a complex prefactor. Both depend on the coupling between the resonant states and the leads connecting the dot to the reservoirs. The phase of the transmission coefficient, given by

$$\theta(t_{\text{QD}}) = \theta_n + \arctan \frac{2}{\Gamma} (E - E_n) \quad (3)$$

changes by π from one side of the resonance to the other (θ_n is the phase of C_n). To find the qualitative behaviour of $|t_{\text{QD}}|$ we measure the magnitude of the AB oscillations, which is proportional to $|t_{\text{QD}}|$ (see equation (1)). As can be seen in Fig. 2c, both magnitude and phase agree well with the prediction of equation (3). These results clearly show that the phase evolution of the transmission coefficient within a resonance peak is correctly described by a Breit-Wigner behaviour.

We now discuss measurements that probe the phase of the transmission coefficient for different CB peaks. Figure 3c shows the phase for a series of five successive peaks seen in Fig. 3a. We find a very similar phase behaviour for all resonances, as suggested by Yacoby *et al.*³. As the plunger gate voltage increases, the QPCs of the QD open slightly because of an electrostatic influence, and coupling to the reservoirs increases. Therefore, the Coulomb peaks widen and overlap, thus reducing the overall phase variation. These results demonstrate unambiguously that successive resonances of a QD have the same phase behaviour.

Another striking feature of the results is the sharp drop of the phase, by π , in the tail of each peak on a scale much smaller than Γ or $k_B T$. We stress that even though this sharp phase change resembles the one observed in a two-terminal measurement that forces rigidity of the phase, its physical origin is different, directly associated with the QD. Figure 3b shows the amplitude of the AB oscillations, which are proportional to $|t_{\text{QD}}|$, as a function of the plunger gate voltage. We see that the phase jump occurs when the oscillation amplitude vanishes, namely when $t_{\text{QD}} = 0$. The fact that $|t_{\text{QD}}|$ vanishes although the conductance of the complete system is not at a minimum (Fig. 3a) is a direct result of interference with the reference arm.

We now briefly discuss the phase behaviour of consecutive resonances. In a simple one-dimensional resonant tunnelling model, one expects the phase of the transmission coefficient to differ by π for each successive resonance. This was not observed in our experiment. For resonant tunnelling through an arbitrary system, the phase of the prefactor in equation (2), $\theta(C_n) = \theta_n$, depends on the overlap between the wavefunctions of the quasi-bound states in the QD and those in the leads, and thus in general is expected to be different for successive resonant states. Because at zero magnetic field time-reversal symmetry holds, the wavefunctions of the quasi-bound states in the QD can be chosen to be real, leading to a phase θ_n with only two possible values^{9,13}: $\theta_n = \theta_0 \pm \pi/2$. When considering the case of a circular⁹ or a rectangular¹⁰ QD, the phase difference of consecutive resonances, $\Delta\theta = \theta_n - \theta_{n+1}$, depends on the symmetry of the QD; successive resonances both in phase ($\Delta\theta = 0$) and out of phase ($\Delta\theta = \pi$) are possible. In our system we do not expect a particular symmetry and thus such single-particle arguments cannot explain why large sequences of resonances (up to 10 peaks) have the same phase. Another approach, taken by Levy Yeyati *et al.*⁸, uses the Friedel sum rule for the case of a QD embedded in one arm of an AB ring. Our results indicate that this model, which predicts a phase shift of π for every added charge e in the whole system (AB ring and QD), is not applicable in the present case.

The appearance of a second energy scale in the phase jump between resonances also cannot be understood as well in a non-interacting picture. Because we find experimentally that each peak has a similar phase behaviour, we model the QD as a sum of displaced Breit-Wigner amplitudes each with the same phase. We find that a phase rise of π within a peak is followed by a phase drop

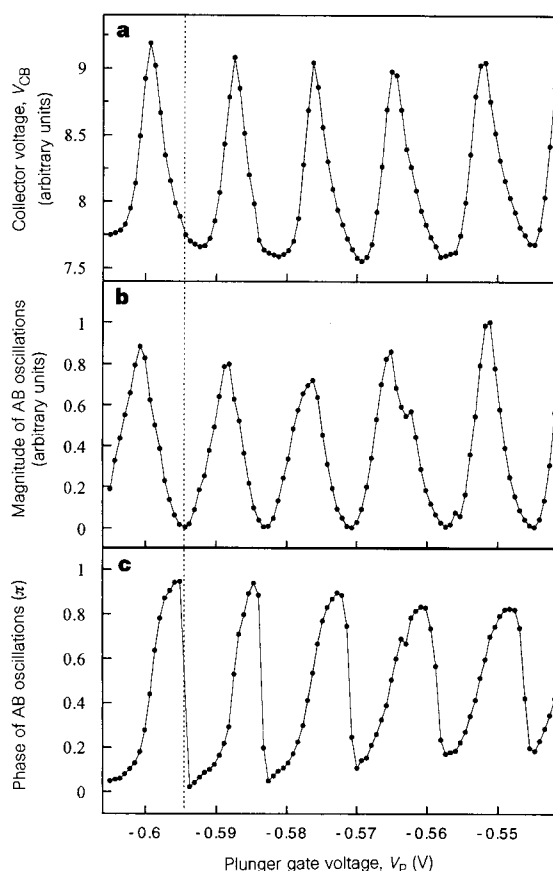


Figure 3 **a**, A series of resonance peaks as a function of plunger gate voltage; **b**, the magnitude of the AB oscillations; and **c**, the corresponding phase all obtained from the interference patterns taken at the marked points. The connecting lines serve only as a guide to the eye. The phase has a periodic behaviour which repeats itself at each resonance. Note the sharp phase jump between resonances.

of π , but the fact that this is on the same energy scale is in clear contradiction to our experimental results. In a recent interference experiment¹³ we measured the phase of the reflection coefficient of a QD. In this experiment, again, the phase behaviour within a resonance could be well described by a single-particle model, but away from the resonance, near the points of minimum conductance, the behaviour, as in this case, is not well understood. □

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Bacterial templating of ordered macrostructures in silica and silica-surfactant mesophases

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The synthesis of inorganic frameworks with specified and organized pore networks is of potential importance in catalysis^{1,2}, separation technology³ and biomaterials engineering^{4,5}. Ordered arrangements of porous channels have been produced in silica-based materials by post-synthetic removal of surfactant templates from inorganic-organic mesostructures^{6,7}. The resulting pore sizes are commensurate with the packing dimensions of the organic molecules, and are currently limited to length scales of up to 10 nm. Here we show how a bacterial superstructure, consisting of a thread of coaligned multicellular filaments of *Bacillus subtilis*^{8,9}, can be used to extend the length scale of inorganic materials patterning. We produce ordered macroporous fibres of either amorphous silica or ordered mesoporous silica^{6,7} (MCM-41) by template-directed mineralization of the interfilament spaces followed by removal of organic material by heating to 600 °C. The inorganic macrostructures consist of a macroporous framework of 0.5- μ m-wide channels with curved walls of either silica or mesoporous silica, 50 to 200 nm in thickness. The formation of ordered pores in the MCM-41 replica on both the mesoscopic and macroscopic length scales illustrates how supra-molecular and supercellular templates might be combined for the fabrication of inorganic materials with structural hierarchy.

Our approach to using a bacterial superstructure to extend the length scale of materials templating in silica-based systems is illustrated in Fig. 1. Individual multicellular filaments of *B. subtilis* are coaligned by slowly drawing a macroscopic thread from a web culture^{8,9}. The superstructure of the bacterial thread resembles the arrangement of hexagonal packing of surfactant cylinders in the surfactant-water H_1 phase¹⁰, except that there is an increase in length scale of two orders of magnitude. In both cases, the interstitial spaces and contact edges represent a continuum for patterned inorganic mineralization. But whereas the 1-nm-wide contact spaces of the surfactant arrays can be readily bridged through the condensation of silicate anions associated with the surfactant head-groups in the synthesis of MCM-41¹⁰, the formation of a coherent wall structure in the much larger void volume of the bacterial superstructure requires extensive *in situ* polymerization. Thus, we use silica-based nanoparticles, rather than molecular species, as the primary building block of mineralization within the 100- to 200-nm wide interfilament spaces.

Previous studies have shown that inorganic minerals can be precipitated on and within *B. subtilis* thread by drawing fibres from web cultures enriched in soluble metal (Ca^{2+} , $Fe(II/III)$, $Cu(II)$) salts and drying in air^{11,12}. To infiltrate the preformed bacterial superstructure with inorganic nanoparticles, we exploited the observation that dried unmineralized bacterial thread swells in water to 1.4 and 1.2 times the original length and width, respectively, without loss of structural integrity¹³. In addition, the swollen hydrated thread contracts to its original dimension when dried in air. We use this reversible swelling property to consolidate the infiltrated nanoparticles so that interconnecting mineral walls are formed within the interfilament spaces (Fig. 1). For example, drying of threads dipped into a silica sol gives rigid white fibres with a

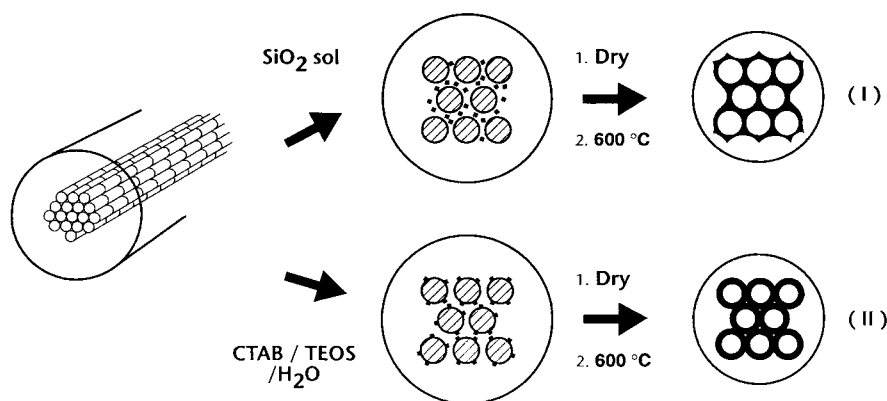


Figure 1 Scheme showing two routes to the formation of organized macroporous inorganic frameworks using bacterial superstructural templates. On the extreme left is a sketch of a dried macroscopic (0.2 mm thick) thread showing ordered superstructure of 0.5- μm -wide multicellular bacterial filaments. Route I: Infiltration and mineralization of the interfilament spaces of swollen thread (viewed in cross-section) with amorphous silica. An air-dried thread is dipped into an aqueous colloidal sol of negatively charged silica nanoparticles. The sol resides primarily in the void spaces of the superstructural array. Drying the composite threads in air results in shrinkage of the thread and compaction of the particles to give an extended inorganic framework. A macroporous replica is subsequently produced

by removal of the organic material by controlled heating. Route II: Infiltration and mineralization of the interfilament spaces with the silica-surfactant mesophase, MCM-41. Air-dried thread is dipped into a synthesis mixture containing an alkaline solution of hexadecyltrimethylammonium bromide (CTAB) and tetraethylorthosilicate (TEOS) for 1 h at room temperature. MCM-41 particles are closely associated with the cell walls of the bacterial filaments. Drying in air for 24 h results in shrinkage of the thread and consolidation of the mineral-coated filaments to give an extended inorganic framework. Controlled heating removes the organic material to produce an inorganic replica with micrometre-size channels comprising walls of mesoporous silica.

striated surface texture running parallel to the fibre axis (Fig. 2). Energy dispersive X-ray (EDX) elemental mapping of thick cross-sections of the mineralized threads indicated complete infiltration of the bacterial fibre when dipped into sols of 30 wt% for 5 minutes (data not shown). Thin transverse sections of the mineralized threads showed an ordered continuous silica framework patterned within the interstitial spaces of the superstructural array (Fig. 3). The continuity and structural integrity of the mineral walls were influenced by the initial concentration of the silica sol; only dispersions with solid contents >15 wt% gave an extended silica architecture that did not significantly fragment on sectioning. The thickness of the silica walls was ~ 200 nm in the void spaces, and 50 nm between the contact edges of adjacent filaments. Local variations in the packing density of the bacterial filaments were reflected in commensurate changes in the mineralized framework such that the architecture did not show long-range periodicity.

Controlled heating of silicified bacterial threads to 600°C resulted in a weight loss of 35% (data not shown). Approximately 8 wt%, corresponding to water, was lost below 200°C . The remaining weight loss between 200 and 600°C was attributed predominantly to organic material. The resulting intact white brittle fibre was composed of amorphous silica and a small residue of organic material and inorganic salts (EDX analysis and Fourier transform infrared spectroscopy; data not shown). Fractured samples showed an ordered macroporous silica framework with uniform 0.5- μm -diameter channels oriented parallel to the fibre axis (Fig. 4). The images were consistent with crack propagation along the poorly mineralized contact edges between adjacent filaments, rather than through the interstitial void spaces that were more densely silicified. The brittle nature of the calcined material resulted in extensive fragmentation of the wall structure when cut in thin sections for examination by transmission electron microscopy (TEM). Although the walls were intact after heating, sintering resulted in partial shrinkage and formation of nanoscopic voids within the silica framework.

We also prepared monolithic silica fibres with ordered porosity at both the meso- and micrometre scale. Structural hierarchy was achieved by replacing the silica sol with a synthesis mixture for the silica-surfactant mesophase, MCM-41. Dried thread was dipped at various times after mixing a stirred alkaline solution of hexadecyl-

trimethylammonium bromide with tetraethylorthosilicate (TEOS), and withdrawn after 60 minutes. Threads dipped 3 minutes after addition of TEOS were coated with a white precipitate, consisting of thin ribbons of hexagonally ordered mesostructured silica (TEM data not shown). The air-dried product was also extensively mineralized throughout the thread such that the multicellular filaments were encased in an extended polycrystalline framework of the silica-surfactant mesophase (Fig. 5a). The mesostructured interfilament walls were often thinner (~ 100 nm) and less ordered than for the pure silica frameworks but generally remained intact during sectioning procedures (Fig. 5).

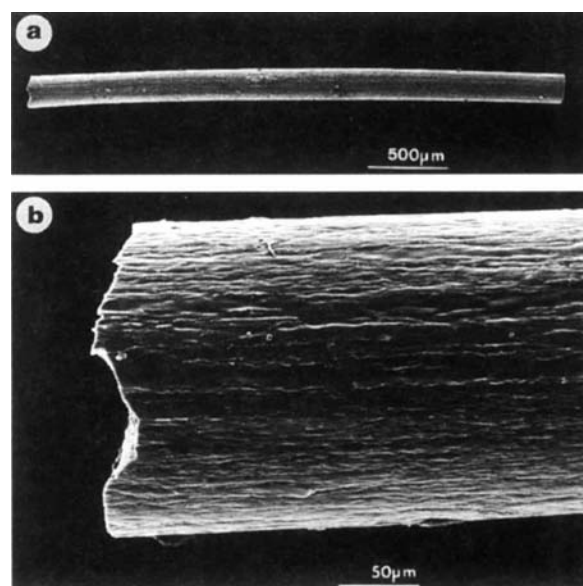


Figure 2 SEM images of air-dried bacterial thread infiltrated with a 30 wt% silica sol. **a**, Low-magnification image showing a mineralized bacterial thread, ~ 250 μm in thickness. **b**, Higher-magnification image of the thread showing surface texture.

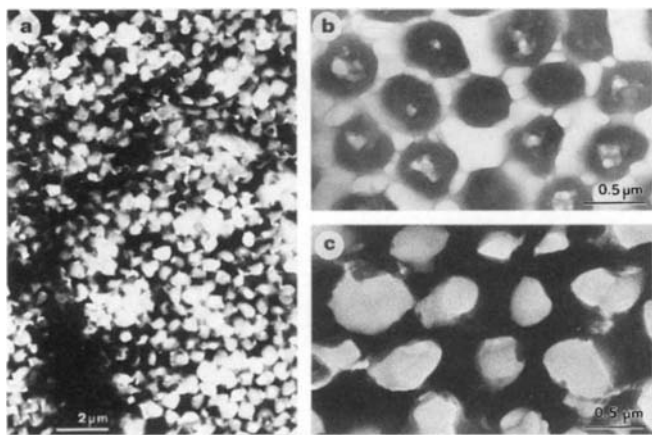


Figure 3 TEM images of sectioned bacterial thread viewed approximately parallel to the fibre axis. **a**, Low-magnification image showing organized macroporous silica replica of the interfilament spaces. **b**, High-magnification image of unmineralized thread showing bacterial filaments and void spaces; **c**, corresponding image of mineralized thread showing formation of continuous silica walls and encapsulated multicellular filaments.

Thermogravimetric analysis of a MCM-41 mineralized thread indicated that water, bacteria and surfactant were removed during heating to 600 °C (data not shown). The resulting intact fibre consisted of a porous framework of coaligned 0.5-μm-wide channels enclosed in walls of mesoporous silica. The mineralized thread appeared to fracture such that discrete hollow cylinders of mesoporous silica remained intact (Fig. 5c), suggesting that the organized composite was assembled from close-packed MCM-41 coated bacterial filaments and not, as in the case of the pure silica material, from the consolidation of weakly associated nanoparticles within the interfilament spaces (Fig. 1).

The difference in these mechanisms can be explained by the negative surface charge of the silica nanoparticles which inhibits association with the highly anionic groups present in the cell wall of Gram-positive bacteria¹⁴. The particles therefore reside primarily in the interstitial void spaces of the superstructural template and thus can permeate deep into the bacterial thread. Indeed, it was difficult to infiltrate swollen bacterial threads with positively charged colloidal sols such as titania and alumina, because of strong binding to the surface of the macroscopic fibre which resulted in uniform external coatings (S.A.D., N.H.M. and S.M., unpublished data). Drying of the silica-containing threads results in large increases in electrolyte concentrations which probably neutralizes the surface charge of the colloidal particles and facilitates the formation of coherent wall structures. In the MCM-41-containing system, there is the possibility that mineralization occurs by either *in situ* precipitation or infiltration of preformed particles, or both. However, in each case, the presence of the cationic surfactant will minimize the number of exposed negatively charged groups and thereby increase the degree of particle aggregation on the cell walls during swelling of the bacterial filaments. This is consistent with the presence of the mineral-coated filaments in the fractured samples, extensive mineralization of the outer surface of the bulk fibre, and requirement for long immersion times (60 minutes) to achieve complete infiltration.

The successful infiltration of bacterial thread with preformed or incipient inorganic nanoparticles, either through associative or non-associative mechanisms, suggests that other biological and synthetic superstructures with reversible swelling properties could have potential uses as templates for the micrometre-scale assembly

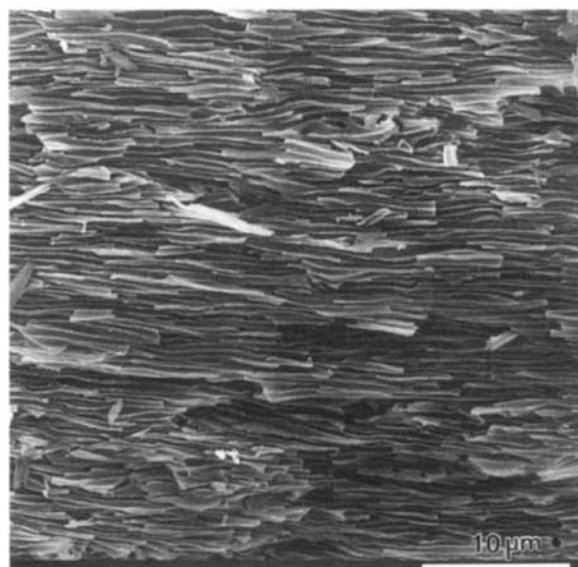


Figure 4 SEM image of a silica-infiltrated bacterial thread after heating to 600 °C and fracturing parallel to the fibre axis. The internal architecture consists of an organized array of uniform sized 0.5-μm-wide silica-walled channels oriented parallel to the fibre axis.

of ordered porous ceramic architectures. A complementary approach is to directly exploit biomineralization processes as biofabrication systems, for example in the production of synthetic 'flat pearls' from living red abalone¹⁵. Alternatively, templates can be produced as transitory architectures generated by microphase transformation and separation processes in self-organized media¹⁶. For example, complex micrometre-scale inorganic patterns and shapes have been synthesized by interfacial templating in microemulsions^{17–19} and vesicle dispersions²⁰. When combined with surfactant templates, this approach produces ordering on two different length scales^{19,20}. Together, these developments suggest an important future role for biotechnology, as well as biomimetics^{21,22}, in the synthesis of organized inorganic-based materials and composites. □

Methods

Preparation of bacterial thread. Decimetre-long bacterial threads were pulled directly from web cultures of *B. subtilis* strain FJ7(II) using a thin wire hook drawn up at 22 mm min⁻¹ by a rate-controlled motor, and left to dry in the air. Cultures were grown at 20 °C in glass filter funnels containing 100 cm³ TB medium (tryptose 10 g l⁻¹, beef extract 3 g l⁻¹, NaCl 5 g l⁻¹) supplemented with uracil (20 μg cm⁻³)²³.

Preparation of silica macrostructures. The tip of a 5-cm length of dried bacterial thread was held between reverse tweezers and the fibre dipped into a silica sol (Syton X30, Monsanto; 3, 6, 15 or 30 wt% solids; mean diameter, 16 nm; pH 9.7) for 5 min at room temperature. The tweezers were not immersed so that the tip of the thread remained dry. This aided withdrawal of the swollen material as an intact composite fibre that was subsequently dried in air for 24 h. Silica-infiltrated bacterial thread was heating to 600 °C on a thermogravimetric balance (Setaram TG-DTA92) at a heating rate of 5 °C min⁻¹. The cooled samples were fractured parallel to the fibre axis and studied by electron microscopy. Mineralized samples were mounted onto Al stubs, using carbon sticky pads and gold coated for scanning electron microscopy (SEM) and EDX analysis. EDX analysis was usually recorded from the surfaces of uncoated samples. Thin sections were prepared as follows. A portion of the mineralized thread was dehydrated for 1 h in 50 cm³ of 50:50 MeOH/H₂O to which 0.5 cm³ silane had been added. After air-drying for 90 min the fibre was transferred to a sample bottle, covered in TAAB hard resin, and placed in a rotator for at least 12 h to allow infiltration of the resin into the fibre. The sample was then placed in an embedding mould and covered with fresh resin

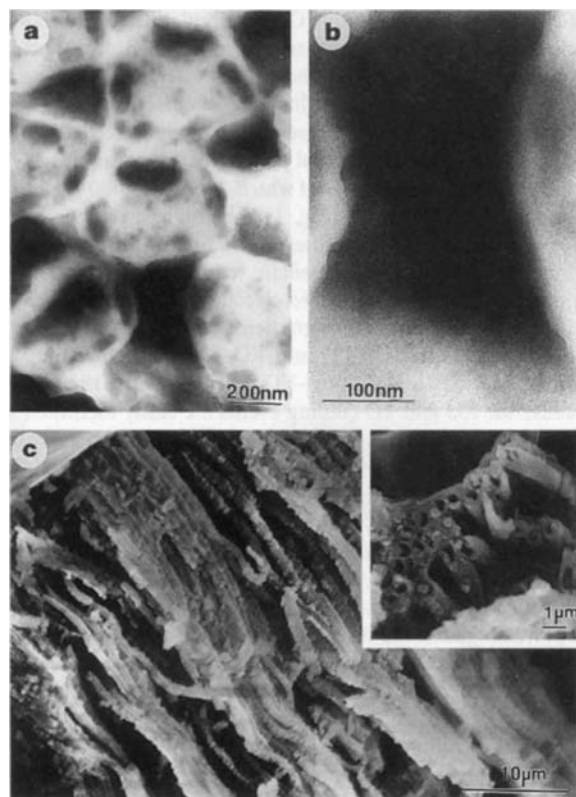


Figure 5 a. TEM image of a thin cross-section of a bacterial thread containing the silica-surfactant mesophase, MCM-41. The superstructural array of multicellular filaments is encased by mesostructured walls of MCM-41. Note also the presence of MCM-41 nanoparticles within the multicellular filaments. **b.** High-resolution image of an individual wall showing periodic lattice fringes (3.5 nm spacing) corresponding to the hexagonally ordered silica-surfactant mesophase. **c.** SEM image of MCM-41 infiltrated bacterial thread after heating to 600°C and fracturing parallel to the fibre axis, showing intact hollow cylinders of mesoporous silica. Inset, transverse view of calcined thread showing hollow MCM-41 tubules.

which was polymerized in an oven at 60°C for 2 d. Thin sections (60–90 nm or 90–150 nm) were cut with a diamond knife perpendicular to the fibre axis. Sections were collected on formvar-covered, carbon-coated 3 mm slotted copper grids and were left unstained. Samples for FTIR were prepared as KBr pellets.

Preparation of MCM-41 macrostructures. An alkaline solution (pH 12) of hexadecyltrimethylammonium bromide (CTAB) was prepared at room temperature by adding 0.36 g to 15.80 g H₂O containing 4.5 g of 1 M NaOH. Tetraethylorthosilicate (TEOS; 1.85 ml) was added with continual stirring. The final molar composition was 0.5 NaOH:0.12 CTAB:130 H₂O:SiO₂. Hydrolysis of TEOS was apparent after 1–3 min when droplets of the reagent were no longer observed. A white precipitate was apparent after 5 min. Under normal synthesis procedures, the product was collected after ageing for 24–48 h. Bacterial thread was dipped into the synthesis mixtures at various times after the addition of TEOS (3, 60, 720 min) and immersed for 60 min at room temperature before removal and air-drying for 24 h. Mineralized threads were heated to 600°C on a thermogravimetric balance (Setaram TG-DTA92) at a heating rate of 5°C min⁻¹. Samples were analysed as described above. Lattice imaging was used to confirm the presence of mesostructured silica in sectioned material.

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Exceptionally steep north-south gradients in lake temperatures during the last deglaciation

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During the transition from the last glacial to the present interglacial climate (late-glacial period), high summer insolation¹ combined with the presence of the Laurentide ice sheet is thought to have promoted the development of strong summer thermal gradients in North America south of the ice margin². Here we use palaeoecological methods to obtain quantitative evidence for the existence of these gradients. Our palaeoclimate reconstructions are based on water temperatures inferred from fossil assemblages of aquatic midge larvae in five lakes along a 240-km transect, extending from central New Brunswick, Canada, to southeastern Maine, USA. We show that the temperature gradient shifted during the Killarney Oscillation (KO) and Younger Dryas (YD) cooling events, and that water temperature differences of 9 and 11 °C existed over distances of 55 and 240 km, respectively, during parts of the late-glacial period. These gradients are much stronger than the current north-south trends of lake summer surface water temperatures for these five lakes and for lakes across the northern tree line. The rapid motion of such steep temperature gradients may have affected the progressive development of aquatic and terrestrial ecosystems in the region.

Although the hypothesized link between midge assemblages and climate was at one time a controversial issue^{3–6}, recent statistical analysis of midge and environmental data of eastern Canadian lakes has shown that the abundance and distribution of midge larvae (Chironomidae and Ceratopogonidae) are closely related to lake summer surface water temperature^{7–9}. A midge-temperature transfer function was subsequently developed which enables estimates of

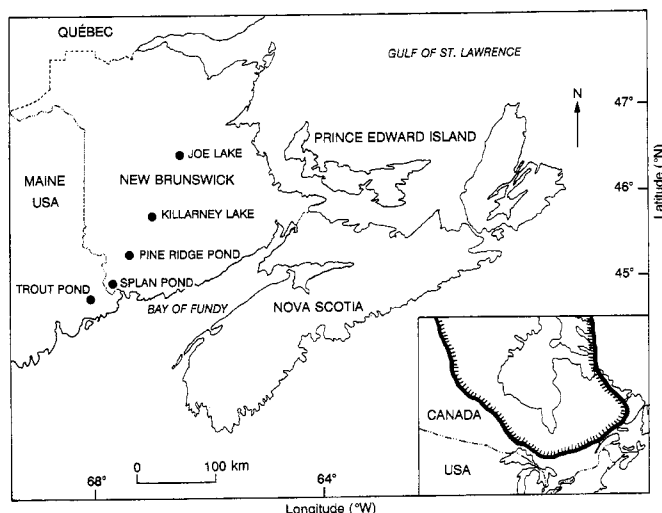


Figure 1 Map showing the location of the five lakes within the study region. The inset (42–68°N, 58–112°W) shows the extent of the Laurentide ice sheet at 10,000 yr BP (thick continuous line), as well as the international border between Canada and the United States (dot-dashed line).

past maximum summer surface water temperatures of lakes to be derived from fossil midge larval assemblages^{7,10}.

To reconstruct late-glacial thermal regimes, we analysed the fossil midge larval remains from five relatively small and shallow sites: Joe Lake (4 ha, 1.75 m deep), Killarney Lake (10.5 ha, 9.8 m), Pine Ridge Pond (1 ha, 5.55 m), Splan Pond (4 ha, 10.6 m) and Trout Pond (3 ha, 11.4 m) (Fig. 1). When the transfer function (recently modified to incorporate an expanded training set¹¹) was applied to the midge assemblages from the five study sites, the resulting late-glacial temperature curves clearly registered the Killarney Oscillation (KO; 11,200–10,900 years before present, yr BP) and Younger Dryas (YD; 10,800–10,000 yr BP) cooling events known to have affected the region^{10–21} (Fig. 2), and which are thought to have been caused by a loss of heat transport to northern latitudes as a

result of changes in North Atlantic Ocean circulation²². The reconstructed late-glacial temperature curves also revealed pronounced north–south thermal gradients. The coldest late-glacial temperatures were consistently recorded at Joe Lake, the northernmost site, where summer surface water temperatures rarely exceeded 17°C (Fig. 2). Throughout the late-glacial period Joe Lake was closest to the margin of the retreating ice sheet which, even at 10,000 yr BP, was nearby in southern Québec^{12,23} (Fig. 1). The highest temperatures (29°C) were recorded at the southernmost site, Trout Pond, with intervening sites exhibiting intermediate temperatures (Fig. 2).

Five points in time within the late-glacial period were chosen in order to reconstruct temperature gradients along the transect: (1) just before the KO (pre-KO), (2) mid-KO, (3) just before the YD (pre-YD), (4) mid-YD, and (5) post-YD. These reference times were used because the KO and YD cooling events are easily identified in the temperature curves, and therefore serve as good benchmarks. Because ice-core^{24,25} and marine²² records show these events to be abrupt, and because the length of the transect is relatively short (240 km), it is assumed that the KO and YD cooling events were not time transgressive, that is, each event affected the five sites simultaneously. The two temperature estimates nearest to each of the reference times (Fig. 2) were averaged, and the average for each site was plotted against the distance of that site along the transect (Joe Lake = 0 km; Fig. 3a). The resulting graphs (Fig. 3a) reveal how temperatures differed along the transect at specified times during the late-glacial period. All sites initially showed progressive but unequal warming following deglaciation (Fig. 2) such that just before the KO (pre-KO) a strong gradient of 11°C had become established (Fig. 3a). The three middle sites differed little but were warmer than the northernmost site and colder than the southernmost site. With the advent of the KO, temperatures of the four northernmost sites declined to ~14°C while Trout Pond exhibited little change. The net result was an exceptionally strong gradient of 9°C across the southern 55 km of the transect (Fig. 3a). From the difference diagram (Fig. 3b), it is evident that intermediate sites cooled the most. During the subsequent brief warm interval (pre-YD point) that separates the KO and YD, all sites experienced greater warmth. Intermediate sites warmed the most (Fig. 3b), resulting in a more or less uniform north–south gradient of 11°C over 240 km (Fig. 3a). The strong cooling of the Younger Dryas, well

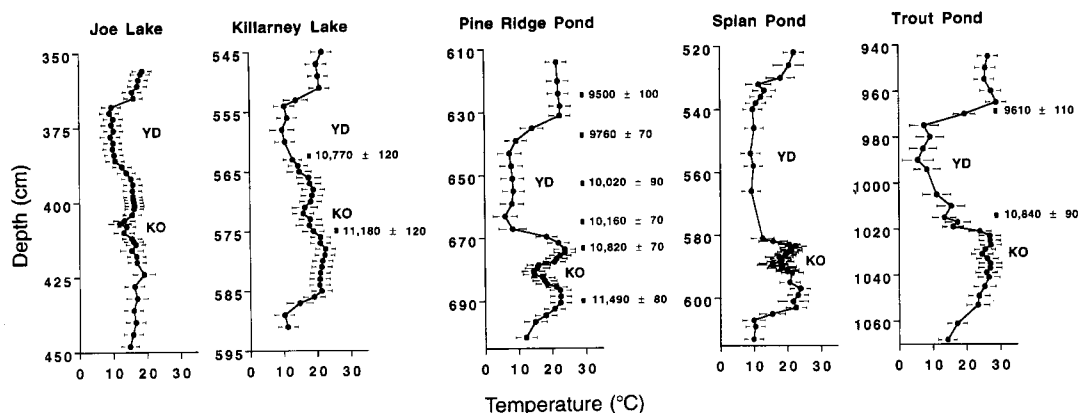
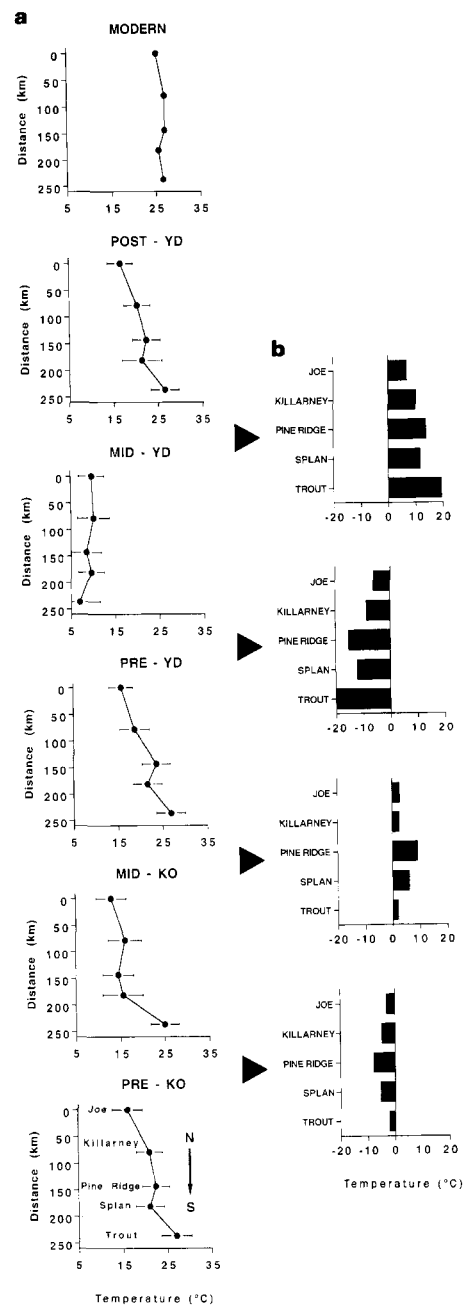


Figure 2 Late-glacial changes in summer surface water temperature at each of the five study lakes. With the exception of Joe Lake, all other diagrams have been previously published^{15,14,15,17}, although the sampling resolution was increased for Killarney Lake and Splan Pond. Reconstructed summer surface water temperatures and associated standard errors of prediction for each of the five sites are available; see Supplementary Information. The Killarney Oscillation (KO) and Younger Dryas (YD) cooling events are registered at all sites. The inferred temperatures and accompanying error estimates (bootstrapped estimates of

the standard error of prediction) were obtained using the computer program WACALIB version 3.2³⁵. The bootstrapped root-mean-square error of prediction is 3.0°C. Filled squares indicate samples submitted for dating and the numbers beside them are the radiocarbon dates obtained as yr BP. Radiocarbon dates for Joe Lake and Splan Pond are not shown because the material used for dating was recovered from cores other than the one used for midge analysis. Detailed information on the radiocarbon dates from each site is available elsewhere^{14,15,17}.

Figure 3 a, Plots showing the gradient in lake summer surface water temperature between Joe Lake and Trout Pond at five different points in time during the late-glacial period. The error bars represent the largest standard error of the two temperature estimates that were averaged. Modern temperatures of the five study sites represent actual temperature measurements. Simple linear regression analyses indicate gradients with significant slopes ($P < 0.05$) for the pre-KO, pre-YD and post-YD time points. No significant gradient existed for the mid-YD time point. Although linear regression did not reveal a significant slope for the mid-KO time point, a least significance difference test indicates that Trout Pond, the southernmost site, was significantly warmer than the other lakes at that time. **b**, Difference diagrams illustrating the magnitude of temperature change at each site between points in time during the late-glacial period. Temperature data appearing in the gradient diagrams (**a**) and in the difference diagrams (**b**) are available; see Supplementary Information.



documented around the North Atlantic seaboard²⁶, and possibly a global event^{27–31}, dramatically reduced temperatures at all sites along the transect by 6–20 °C, with the absolute amount of cooling increasing southwards (Fig. 3b). As a result, the north–south temperature gradient disappeared as water temperatures centred around 9 °C (Fig. 3a). This does not imply that a steep temperature gradient did not exist at that time, but rather that any existing gradient was probably shifted south beyond the limits of the transect owing to the severe cooling of the Younger Dryas. The warming that terminated the Younger Dryas (post-YD) and began the Holocene epoch (present interglacial period) resulted in the re-establishment of a strong gradient of 10 °C (Fig. 3a) as all sites warmed, but with progressively larger increases in water temperature southwards along the transect (Fig. 3b). Modern water temperatures (Fig. 3a) are uniformly high for all sites (25–27 °C) with no apparent gradient.

Pollen and macrofossil studies^{13–21} indicate that, with the possible exception of the mid-YD time period, the position of the late-glacial tundra/forest ecotone (vegetation transitional between tundra and forest) in eastern North America lay between Joe Lake and Trout Pond, and was therefore closely associated with late-glacial temperature gradients. For the purpose of comparison, we examined the lake water temperature gradient across the modern tree line using temperature data collected from 70 lakes spanning tundra, forest tundra, lichen woodland, forest and coastal barren areas in Labrador³² (Fig. 4). Regression analyses demonstrated that log(depth) alone could account for 28% of the variance in Labrador lake temperatures, whereas latitude (our best proxy of climate) could account for 68% of the variance. The modern lake-temperature variability at a given latitude (Fig. 4) is mostly attributable to variations in lake depth. The regression of temperature against latitude of Labrador lakes also indicated a summer surface water temperature gradient across the modern tree line of 1 °C per 100 km.

Temperature differences across the late-glacial tundra/forest ecotone ranged from 9 to 11 °C over distances of 55 to 240 km, respectively, resulting in remarkably strong gradients of 16.0 °C per 100 km (mid-KO) to ~4.6 °C per 100 km (pre-KO, pre-YD and post-YD). In addition, using the temperature–depth regression for Labrador lakes we have determined that, due to differences in depth alone, the expected temperature of Joe Lake (late-glacial depth, ~5.75 m) would have been warmer than Trout Pond (late-glacial depth, ~21.4 m) by ~1.8 °C. These results imply not only that the increase in water temperature between Joe Lake (northernmost site) and Trout Pond (southernmost site) during the late-glacial period must be the result of differences in climate along the transect, but also that the magnitude of reconstructed late-glacial temperature gradients are probably underestimates. We note that some of the variance in lake water temperatures along the transect may also be due to secondary climatic effects such as inflowing meltwater streams or melting out of ground ice during thermokarst activity

(ground movement caused by heating of permafrost). However, none of our sites, especially the northernmost ones, show large influxes of taxa characteristic of glacial melt water (that is, subfamily Diamesinae³³) or of terrestrial/semi-terrestrial types that would be eroded into the lake as a result of thermokarst activity.

These strong gradients arose from the unique conditions that existed in New Brunswick during the late-glacial period. The amount of incoming solar radiation at a latitude of 45° N in July between 12,000 and 10,000 yr BP was 9% higher than at present¹. However, during the late-glacial period the southern margin of the ice sheet was only slightly north of Joe Lake. The combination of a narrow chill zone south of the ice margin, and higher than present summer insolation values for the study region, would have caused the development of the strong thermal gradients observed in the region during the late-glacial period. Subsequent collapse of the gradient from early Holocene times to the present remains to be studied, but is undoubtedly related to the continued withdrawal of the Laurentide ice sheet northward and its eventual disintegration

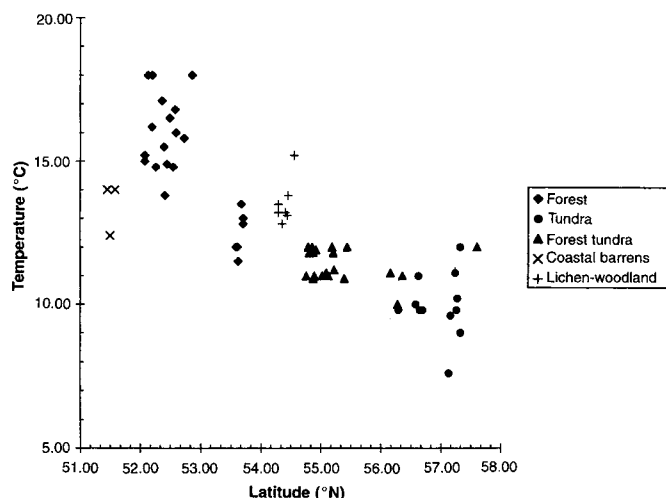


Figure 4 Scatter plot of actual summer surface water temperature versus latitude for each of the 70 Labrador lakes. The different symbols represent the different vegetation zones in which the Labrador lakes are located³². Temperature, depth, latitude and longitude of the lakes are available; see Supplementary Information.

about 6,500 yr BP, as well as decreases in the amount of summer season radiation.

The temperature gradients reconstructed for the late-glacial period in eastern North America are very strong relative to those of modern times. Under these conditions, plant species whose modern ranges are normally separated by large distances may have occurred in close proximity. These results may help explain, in some cases, the lack of modern analogues for past pollen assemblages, typified by those comprising pollen of spruce (*Picea*), sage (*Artemisia*), ragweed (*Ambrosia*), oak (*Quercus*), elm (*Ulmus*) and ash (*Fraxinus*) during the late-glacial period in Minnesota³⁴. Our results also indicate that the rapid warming and cooling phases of the Killarney Oscillation and Younger Dryas affect terrestrial ecosystems by rapidly changing the position of steep temperature gradients, to which plant and animal communities alter their geographical boundaries accordingly. This would have proved especially difficult for people inhabiting the region at that time, as they would have been subjected to a number of severe and abrupt climate changes throughout the late-glacial period. The late-glacial temperature gradients reported here will serve as a robust test for future, more refined climate models, especially because the magnitude of these gradients is dependent upon the combined effects of the Laurentide ice sheet and increased summer season radiation, the latter resulting from long-term changes of the Earth's orbit relative to the Sun. □

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Supplementary Information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from Mary Sheehan at the London editorial office of *Nature*.

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Direct measurement of *in situ* methane quantities in a large gas-hydrate reservoir

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Certain gases can combine with water to form solids—gas hydrates—that are stable at high pressures and low temperatures^{1,2}. Conditions appropriate for gas-hydrate formation exist in many marine sediments where there is a supply of methane. Seismic reflection profiles across continental margins indicate the frequent occurrence of gas hydrate within the upper few hundred metres of sea-floor sediments, overlying deeper zones containing bubbles of free gas^{3–9}. If large volumes of methane are stored in these reservoirs, outgassing may play an important role during climate change^{10–12}. Gas hydrates in oceanic sediments may in fact comprise the Earth's largest fossil-fuel reservoir^{2,13}. But the amount of methane stored in gas-hydrate and free-gas zones is poorly constrained^{12–9,13–18}. Here we report the direct measurement of *in situ* methane abundances stored as gas hydrate and free gas in a sediment sequence from the Blake ridge, western Atlantic Ocean. Our results indicate the presence of substantial quantities of methane (~15 GT of carbon) stored as solid gas hydrate, with an equivalent or greater amount occurring as bubbles of free gas in the sediments below the hydrate zone.

The Blake ridge is a sediment drift deposit in the Atlantic Ocean on the continental rise of North America that has strong indications for the presence of gas hydrate and free gas on the basis of seismic reflection profiles (Fig. 1)^{2,6–8,18,19}. Ocean Drilling Program (ODP)

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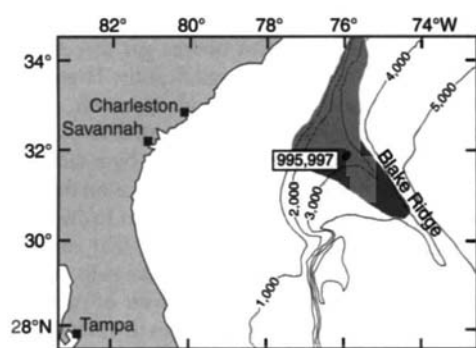


Figure 1 Location of Ocean Drilling Program (ODP) sites 995 and 997 on the Blake ridge off the southeastern coast of the United States²⁰. The region (26,000 km²) over which seismic reflection profiling indicates the presence of gas-hydrate and free-gas zones is shaded¹⁹. Bathymetric contours are in metres.

Leg 164 recently drilled locations on this ridge to investigate *in situ* characteristics and amounts of gas stored in these zones²⁰. Sites 995 and 997 were drilled from the sea floor at approximately 2,775 m below sea level (m.b.s.l.) to depths of 705 and 750 m below sea floor (m.b.s.f.), respectively.

Proxy techniques for identifying the presence of gas hydrate and free gas (seismic profiles, well-logs, porewater chemistry and core temperature) indicate that there are three general gas zones in sediment at sites 995 and 997 (Fig. 2)^{18,20}: (1) sediment is increasingly gassy but does not contain methane hydrate from 0 to 190 m.b.s.f.; (2) sediment contains intervals with methane hydrate from 190 to 450 m.b.s.f. (principally between 190 and 240 m.b.s.f. and between 380 and 450 m.b.s.f.) (3) sediment contains free methane bubbles below 450 m.b.s.f. (especially between 450 and 470 m.b.s.f.). However, as with previous indirect approaches^{3–9,14–17}, how much methane exists in each of these depth intervals is unclear.

The Pressure Core Sampler (PCS) is a tool designed to recover a 1,320 cm³ cylindrical sediment core at *in situ* pressure (Fig. 3)²¹. Thus, the quantity and composition of gas in a deep sea sediment sample at depth can be determined by releasing gas from the PCS under controlled laboratory conditions at the surface. Using the PCS, *in situ* gas quantities were determined at 17 depths at sites 995 and 997 (Fig. 2). Analyses of gas released from the PCS indicate that it is >98.5% CH₄, with the remainder consisting largely of CO₂ (ref. 20).

The downhole profile of *in situ* methane quantities (Fig. 2) is broadly consistent with shipboard interpretations concerning the distribution of gas at sites 995 and 997. Sediment from 0 to 190 m.b.s.f. and from 240 to 380 m.b.s.f. contains relatively low quantities of methane (0.04–0.14 mol dm⁻³ of pore space); sediment from 190 to 240 m.b.s.f., from 380 to 450 m.b.s.f., and from below 470 m.b.s.f. contains moderate quantities of methane (0.23–0.84 mol dm⁻³ of pore space); sediment from 450 to 470 m.b.s.f. contains an exceptional quantity of methane (2.0 mol dm⁻³ of pore space). Theoretical saturation of methane in pore water depends on whether pore space also contains methane hydrate or free methane gas^{1,22–24}. Methane saturation in sea water/methane hydrate systems increases from approximately 0.05 to 0.17 mol CH₄ per dm³ sea water at water depths from 2,775 to 3,225 m.b.s.l. (0–450 m.b.s.f.), whereas methane saturation in seawater-free methane gas systems decreases from approximately 0.17 to 0.16 mol CH₄ per dm³ sea water at water depths from 3,225 to 3,525 m.b.s.l. (450–750 m.b.s.f.)^{22–24}. Thus, pore water is undersaturated with methane in sediment from 0 to 190 m.b.s.f., and saturated to greatly oversaturated in sediment beneath 190 m.b.s.f. (Fig. 2).

A volume of methane hydrate releases a quantity of methane

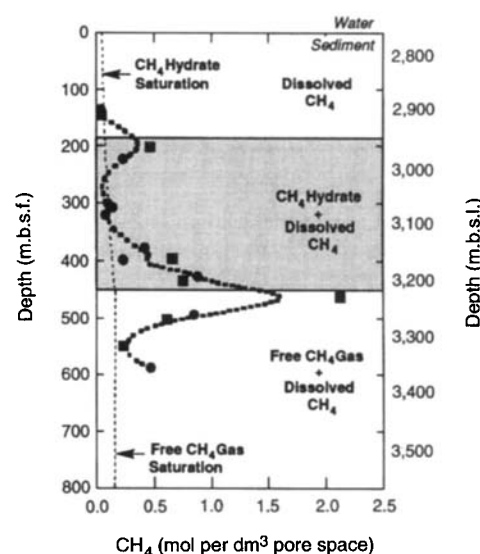


Figure 2 *In situ* methane quantities at Ocean Drilling Program (ODP) sites 995 (filled circles) and 997 (filled squares) shown with respect to water depth (m below sea level, m.b.s.l.) and sediment depth (m below sea floor, m.b.s.f.). Sites 995 and 997 are located at approximately 2,775 m.b.s.l. and extend from the sea floor to 705 and 750 m.b.s.f., respectively. Methane hydrate occurs in sediment from 190 to 450 m.b.s.f. while free methane gas occurs in sediment below 450 m.b.s.f. *In situ* methane quantities (mol per dm³ pore space) were calculated by converting the total volume of gas released from the Pressure Core Sampler (PCS) to moles (23.6 l mol⁻¹ at 1 atm and 15°C), and dividing this amount by the volume of sediment pore space inside the PCS. Sediment porosity used in this calculation was the average value measured for sediment recovered by conventional coring techniques within 10 m of each PCS core. The small volume of gas that remains in the PCS at 1 atm and 15°C cannot be determined and has not been included in these calculations. Also shown are a smoothed curve through all *in situ* methane quantities, and the saturation curves for methane in sea water with methane hydrate^{22,23} and in sea water with free methane gas²⁴.

upon dissociation that is proportional to its density (0.91 g cm⁻³) and molecular weight (124 g mol⁻¹). Assuming that methane in oversaturated pore water exists as methane hydrate above 450 m.b.s.f., pore space from 190 to 450 m.b.s.f. contains between 0 and 9% methane hydrate by volume. This range is quantitatively consistent with shipboard estimates made from porewater Cl⁻ profiles given certain assumptions regarding *in situ* porewater composition²⁰. Methane hydrate volumes from 190 to 450 m.b.s.f. on the Blake ridge also have been estimated from vertical seismic profiling¹⁸. This latter approach gives a similar average value for methane hydrate volumes but a smaller range in values (5–7% pore space) probably because vertical seismic profiling cannot detect the centimetre to metre-scale heterogeneity of methane hydrate distribution that is evident in sediment on the Blake ridge²⁰.

There should be a unique depth along the geotherm in a sediment sequence at which both solid methane hydrate and free methane gas are in equilibrium^{2,12,14,15}. Above this depth, only methane hydrate and methane-saturated pore water should be present; below, only free methane bubbles and methane-saturated pore water. Seismic, well-log and porewater information at sites 995 and 997 indicate that this phase boundary occurs at ~450 m.b.s.f. (Fig. 2)^{18,20}. Moreover, pore waters collected from PCS cores from ≥462 m.b.s.f. do not contain local freshwater anomalies that are produced when gas hydrate dissociates^{2,17,20}. Thus, oversaturated methane in PCS cores from ≥462 m.b.s.f. was present as free gas bubbles.

Free methane volumes at depth can be calculated from methane quantities measured at 1 atm and 15°C via the ideal gas law and *in*

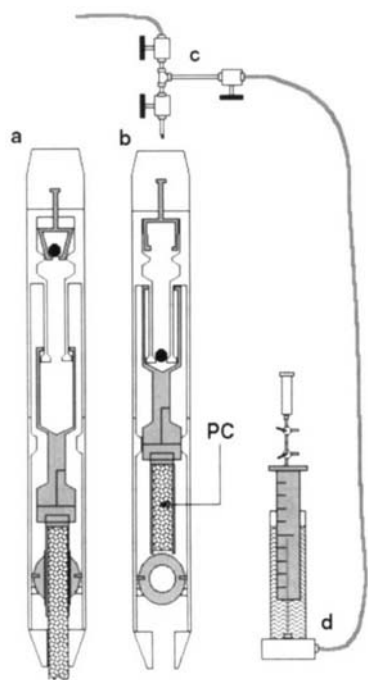


Figure 3 Diagram showing the Pressure Core Sampler (PCS) tool during downward drilling (a) and after core recovery (b) with attached gas manifold system (c) and bubbling chamber (d)^{20,21}. Recovery of a pressurized core of up to 1,320 cm³ (1 m long and 4.1 cm diameter) occurs when an actuation ball is released to redirect fluid flow, to raise a piston with core barrel, and to rotate a lower ball valve. To measure *in situ* gas volumes, the PCS was placed in an ice-bath on ship after each successful core recovery. Gas was released incrementally from the PCS into a He purged manifold (c) that was at ~15 °C and 1 atm and then bubbled into an inverted graduated cylinder (d) that was immersed in water saturated with NaCl at ~15 °C. Aliquots of gas were then removed from the inverted cylinder for analysis. This procedure was continued until atmospheric pressure was achieved in the PCS. The PCS was warmed to ~15 °C and an additional small volume of gas was measured. The volume of sediment inside the PCS then was measured after opening the tool. Repeated tests conducted during ODP Leg 164 have shown that all gas released from the PCS comes from the sediment core inside it, and not from any bore-hole water it might contain²⁰. Detailed descriptions of the PCS and historical efforts at recovering deep-sea sediment cores under pressure are reported elsewhere^{21,27}.

situ pressure and temperature. Sediment at 462 m.b.s.f. contains an oversaturated *in situ* methane quantity of 1.8 mol CH₄ per dm³ pore space (Fig. 2) and has *in situ* pressure and temperature conditions of ~323 atm and 20 °C (ref. 20). Therefore, pore space at this depth contains ~12% methane gas bubbles by volume. This direct evidence indicates that volumes of free gas beneath the gas-hydrate zone on the Blake ridge are significantly higher than have been previously estimated (1–2%) on the basis of seismic reflection profiles and a series of assumptions concerning acoustic properties in gas-rich sediment⁷. Even the data from vertical seismic profiling conducted during Leg 164 have been interpreted as indicating only small amounts of free gas (~1% pore space) beneath the gas-hydrate zone¹⁸, underscoring the need for direct measurements of the type we report here.

The average quantity of gas in PCS cores recovered between 190 and 600 m.b.s.f. at sites 995 and 997 is 0.55 mol CH₄ per dm³ pore space. Seismic reflection profiles indicate that gas-hydrate and free-gas zones extend over 26,000 km² of the Blake ridge (Fig. 1)¹⁹. If results presented here are representative of this entire area, gas-hydrate and free-gas zones on the Blake ridge contain approximately 4.7×10^{16} g CH₄, or 35 GT of carbon. Our estimate differs from that

recently made from vertical seismic profiling (40 GT of carbon over 100,000 km²)¹⁸ in that the amount of free gas directly measured beneath the gas-hydrate zone is significantly larger than that estimated from the indirect geophysical approach. As a consequence, the amount of gas that was estimated to occur in these holes by geophysical measurements is lower by a factor of three. Indeed, the volume of methane present as free gas on the Blake ridge may be greater than that stored in overlying gas hydrate (Fig. 2).

Although we agree with Holbrook *et al.*¹⁸ that some previous estimates of gas hydrate amounts on the Blake ridge are too high, reassessment of the size of the global reservoir of methane in gas hydrate and free gas must await future studies in other regions using direct measurement techniques such as those presented here. There is no indication from our results, however, to suggest that global estimates of 10³–10⁴ GT of methane carbon are in error or that methane hydrate and free-methane zones in marine sediment constitute relatively minor carbon reservoirs. The 35 GT of methane carbon on the Blake ridge is a quantity of methane that could meet the 1996 United States natural gas consumption rate for the next 105 years²⁵, and a quantity of carbon that is ~7% of total terrestrial biota²⁶.

Abundant geological evidence indicates that rapid drops in sea level and deep ocean warming have occurred in the past. Because several of these time intervals are characterized by a major input of carbon (from some source) to the ocean and atmosphere, and because the stability of gas hydrate decreases with reduced pressure and elevated temperature, it has been speculated that release of methane from oceanic hydrates has played a role in past climate change^{10–12}. A major assumption behind such hypotheses has been that a large mass of methane indeed exists in oceanic gas-hydrate zones. Results presented here validate this assumption (at least for the present-day Blake ridge). Release of large quantities of methane from free-gas zones also should be considered in any scenario that invokes dissociation of overlying methane hydrate during oceanographic change. □

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A new Late Eocene anthropoid primate from Thailand

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The fossil record of anthropoid primates from the Middle Eocene of South Asia is so far restricted to two genera (*Pondaungia cotteri* Pilgrim, 1937 and *Amphipithecus mogaungensis* Colbert, 1937 from the Eocene Pondaung deposits of Burma) whose anthropoid status and phylogenetic position have long been under debate^{1–6} because they represent the oldest highly derived fossil primates of anthropoid grade. Moreover, several new African taxa^{7–10}, some of which are even older, have been recently included in the suborder Anthropeoidea, suggesting an African origin for this group. Conversely, new fossil primates recently discovered in China (*Eosimias*) have been related to the most primitive representatives of Anthropeoidea, alternatively suggesting an Asian origin and a probable Asian radiation centre¹¹. We report here the discovery of a new anthropoid from the Thai Late Eocene locality of Krabi^{12,13}, which displays several additional anthropoid characters with regard to those of the Eocene Burmese genera. This species, which is about the size of the Fayum *Aegyptopithecus*, can be related to the Burmese forms, and it further provides strong additional evidence for a southeast Asian evolutionary centre for anthropoids.

Order Primates Linnaeus, 1758
Suborder Anthropeoidea Mivart, 1864
Family ? Propliopithecidae Straus, 1961
Siamopithecus eocaenus gen. et sp. nov.

Holotype. Right maxilla fragment preserving P³–M³, TF 3635 (collections of the Department of Mineral Resources, Bangkok; Fig. 1a).

Horizon and locality. Lower level of main lignite seam of Bang Mark pit, Krabi mine, southern Thailand for the holotype. The referred lower jaw is from Wai Lek pit, Krabi mine, some 400 m South of the Bang Mark pit. Both specimens have been found in the same main lignite seam that occurs in the Krabi mine. The mammalian fauna associated with primate remains is identical in the two pits, and faunal evidence indicates a Late Eocene age¹² and affinities with the Pondaung locality.

Referred material. Right mandibular fragment with distal half of M₁, and M_{2,3} (TF 3634; Fig. 1b and c).

Diagnosis. Large Eocene anthropoid primate (Table 1) of the size of the Burmese *Pondaungia cotteri*, characterized by a mosaic of primitive (P³ smaller than P⁴, paracone larger than protocone, moderately developed cingulum-derived hypocone which is related by a crest to the protocone, centrally situated small hypoconulid on M₁, M₃ with reduced and mesially located entoconid) and derived characters (curved upper tooth row, upper premolars with rounded distal wall, well defined postprotocrista, M₁ larger than M₂, high molar cusp elevation, absence of conules, very slanted lingual and labial walls, and very reduced or absent lingual cingulum, lower molars lacking a paraconid, constriction of the middle of the crown, very weak labial cingulid, great talonid length relative to trigonid length, talonid width narrower than trigonid, reduced and labially located hypoconulid on M₂, and small and mesial entoconid, wrinkled enamel).

S. eocaenus differs from other Asian Eocene primates (*Eosimias*¹¹,

Table 1 Dental measurements (in mm) of *siamopithecus eocaenus*

		Length	Width
TF 3635	right P ³	4.3	5.3
	right P ⁴	5.0	6.5
	right M ¹	5.4	8.1
	right M ²	4.8	8.1
	right M ³	4.2 (lingual part)	6.0 (minimum)
TF 3634	right M ₁	–	5.7 (talonid)
	right M ₂	6.5	5.7
	right M ₃	8.1	5.1

Length M₂–M₃, 13.2; length P³–M³, 21.2.

*Wailekia*¹³, *Hoanghoni*¹⁴, *Rencunius*¹⁵, *Lushius*¹⁶, *Asiomomys*¹⁷) in its deeper horizontal ramus and larger size, its overall structure of both upper and lower molars (flared upper molars lacking a pericone, lower molars with no salient hypoconulid, no labial cingulid, wrinkled enamel). It differs from all Fayum anthropoids^{8–10} in its larger size (except *Aegyptopithecus*), its central lower molars constriction (except *Apidium*, *Parapithecus*, and *Catopithecus*) with a tendency to reduce hypoconulid, in lacking a cingulid, its upper molars with small hypocone linked to the protocone by a crest, strongly slanted buccal and lingual walls without lingual cingulum and lacking accessory cusps, and its crenulated enamel. It differs from *Amphipithecus* in its M₂ with more inwardly situated cusps, with transversely narrower trigonid and talonid and the presence of a distinct hypoconulid, and absence of paraconid. It differs from *Pondaungia* by the absence of paraconid, its lower molars with trigonid shorter than talonid and with a distinct median hypoconulid, and in its M₁/M₂ proportions, M₁ being slightly larger than M₂. It differs from *Wailekia*¹³ and *Eosimias*¹¹ mainly in its larger size, its deeper horizontal ramus, its more bunodont lower molars, its weak hypoconulid on M_{1,2}, and in lacking a labial cingulid.

Height of horizontal ramus under M₃ = 19.5; height of Md/length M_{2,3} is 1.33, whereas it is 1.19 in *Pondaungia* and 1.14 in *Aegyptopithecus*.

The estimated body weight of *Siamopithecus*, based on extrapolated M₁ dimensions¹⁸, is between 6.5 and 7 kg (comparable to the extant South American mantled howler monkey *Alouatta palliata*). Estimates based on M₁ dimensions¹⁹ give similar weights (~6.8 kg). The crowns of the molars are very high but the molar crests of *Siamopithecus* are low and its cusps are rather low and massive, suggesting a diet primarily based on hard food.

Siamopithecus is a large primate whose anthropoid grade is supported by numerous features (deep lower jaw, strong bunodonty, cingulum-derived hypocone, trigonid about the same height as talonid, M₃ smaller than M₂ (in surface), cristid obliqua mesially directed and almost continuous, tendency to reduction and/or disappearance of hypoconulid, absence of upper molars buccal cingulum, well developed hypoparacrista²⁰, almost continuous crista obliqua, and shortness of trigon basin), and whose most peculiar specialization is the rather high and slanted buccal and lingual walls of lower and upper molars (the two latter features are also unknown in any adapiform or omomyid and are considered as anthropoid-derived characters by Godinot and Mahboubi⁷). This molar structure facilitates concentration of the forces during chewing motions on a very small surface, thus increasing the chewing power. Although this specialized molar structure can obscure the phylogenetic relationships of *Siamopithecus*, its overall morphology may help to understand that of the more or less contemporaneous Asian forms (*Eosimias*, *Pondaungia* and *Amphipithecus*). The main problem set by the Thai species concerns the phylogenetic status of its ancestor: an African anthropoid, an Asian anthropoid, or even an Asian adapid. Indeed, although the anthropoid grade of *Siamopithecus* seems to be well established, its attribution to any clade is relatively difficult. Nevertheless, the set of characters observed on the molars of *Siamopithecus*, *Pondaungia* and, to a

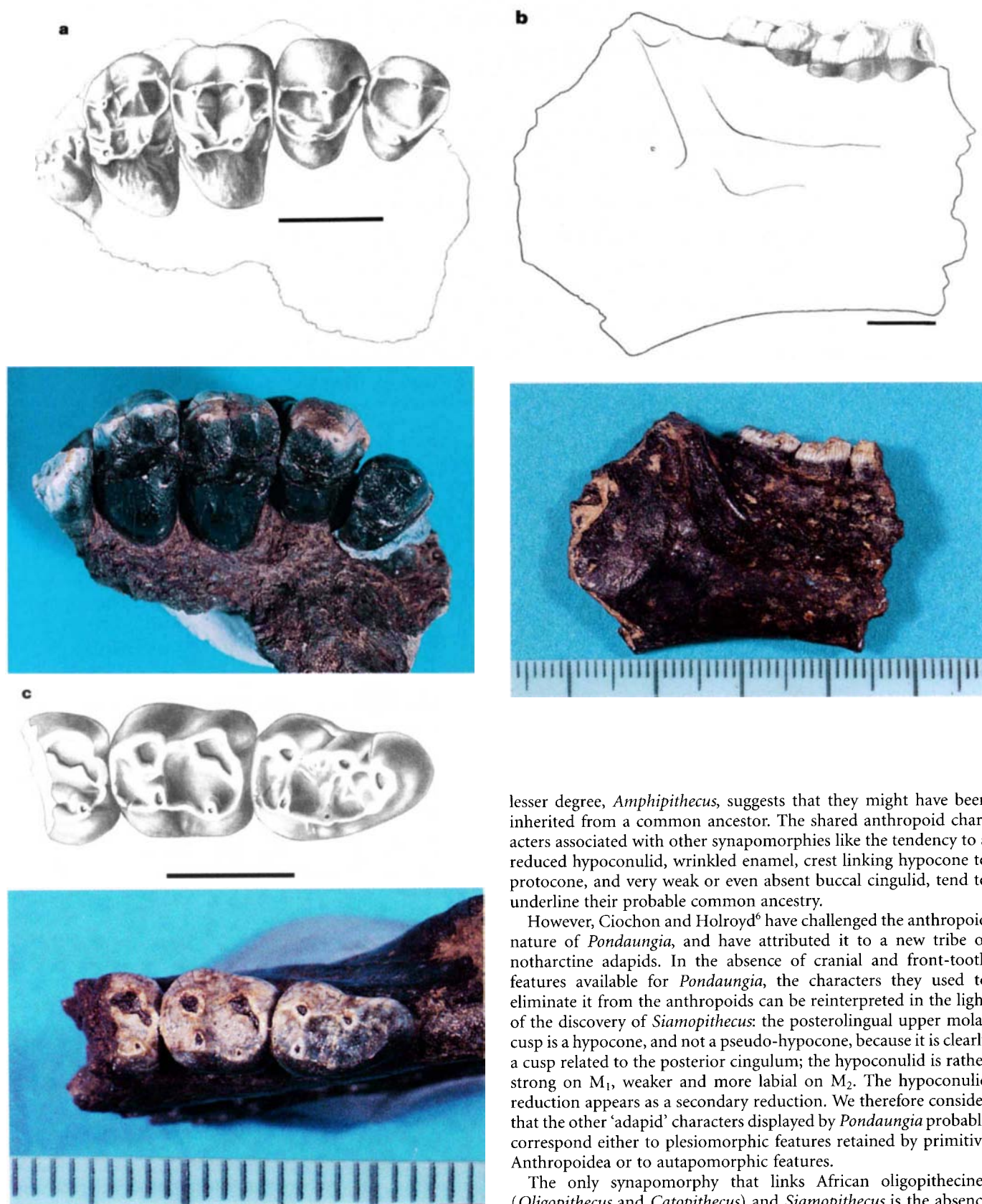


Figure 1 *Siamopithecus eocaenus* gen. et sp. nov. **a**, Occlusal view of the holotype TF 3635 (right P³-M³); the crown of M³ is slightly oblique with respect to the preceding molars, so that it appears smaller than it is (see measurements); **b**, labial view of the mandible; **c**, occlusal view of right M₁₋₃ (TF 3634); scale bars, 5 mm. On the photographs, each division on the scale corresponds to 1 mm (scale in **a** is the same as in **c**). (Drawings by L. Meslin; photographs by J.-Y. Quéro.)

lesser degree, *Amphipithecus*, suggests that they might have been inherited from a common ancestor. The shared anthropoid characters associated with other synapomorphies like the tendency to a reduced hypoconulid, wrinkled enamel, crest linking hypocone to protocone, and very weak or even absent buccal cingulid, tend to underline their probable common ancestry.

However, Ciochon and Holroyd⁶ have challenged the anthropoid nature of *Pondaungia*, and have attributed it to a new tribe of notharctine adapids. In the absence of cranial and front-tooth features available for *Pondaungia*, the characters they used to eliminate it from the anthropoids can be reinterpreted in the light of the discovery of *Siamopithecus*: the posterolingual upper molar cusp is a hypocone, and not a pseudo-hypocone, because it is clearly a cusp related to the posterior cingulum; the hypoconulid is rather strong on M₁, weaker and more labial on M₂. The hypoconulid reduction appears as a secondary reduction. We therefore consider that the other 'adapid' characters displayed by *Pondaungia* probably correspond either to plesiomorphic features retained by primitive Anthropeoidea or to autapomorphic features.

The only synapomorphy that links African oligopithecines (*Oligopithecus* and *Catopithecus*) and *Siamopithecus* is the absence of a paraconule on M¹⁻². Comparisons with the Fayum parapithecids (including *Parapithecus*, *Apidium*, *Simonsius*, *Qatrania*, *Serapia* and *Arsinoea*) reveal several synapomorphies among those listed by Kay and Williams²¹: upper premolars with rounded distal crown margins, lower molars with very low cusp relief and weakened and rounded cristid obliqua, and M₃ talonid reduced in breadth com-

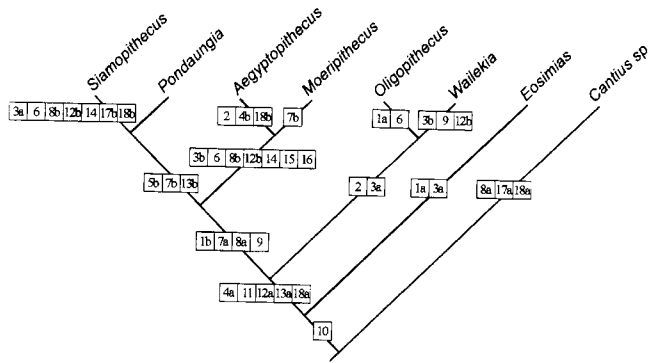


Figure 2 Inferred cladogram of early anthropoid relationships, based on the analysis of 18 dental traits. Derived characters states (squares) are numbered below, and shared derived features appear on the cladogram. Only characters of M_{1-3} and P^3-M^3 have been used. Although it shows strong phylogenetic affinities with *Siamopithecus* and *Pondaungia*, *Amphipithecus* is excluded from the cladogram because only its M_2 is available for the cladistic analysis. 1a, moderately deep lower jaw; 1b, very deep lower jaw; 2, no central lower molar constriction; 3a, small hypoconulid on M_{1-2} ; 3b, strong hypoconulid on M_{1-2} ; 4a, small hypocone on upper molars; 4b, strong hypocone; 5a, weak crest linking hypocone and protocone on upper molars; 5b, strong crest linking hypocone and protocone on upper molars; 6, loss of conules on upper molars; 7a, weak flaring of cheek teeth; 7b, strong flaring on cheek teeth; 8a, slight bunodonty; 8b, strong bunodonty; 9, trigonid about the same height as talonid; 10, M_3 surface $\leq M_2$ surface; 11, cristid obliqua mesially directed; 12a, small paraconid on lower molars; 12b, no paraconid; 13a, weak upper molar buccal cingulid; 13b, no upper molar buccal cingulid; 14, well developed hypoparacrista; 15, lower molar distolingual fovea; 16, P^3 about the same size than P^4 ; 17a, weak lingual cingulum on upper molars; 17b, no lingual cingulum; 18a, body size between 500 g and 5 kg; 18b, body size > 5 kg.

pared with the trigonid. Also, the derived features shared between *Siamopithecus* and the propliopithecine *Aegyptopithecus* are the loss of the molar paraconid, the rounded distal crown margins of upper premolars, and the occurrence of lateral posterior transverse cristae on M^{1-2} . However, some characters separate *Aegyptopithecus* from the new Thai anthropoid. In *Aegyptopithecus* and *Moeripithecus*, the hypocone and lingual cingulum are stronger, P^3 is higher and stronger, the protocone of P^3 and P^4 is more developed, the hypoconulid is salient, median and stronger, the trigonid is mesiodistally short, and the talonid basin is very large with a distinct fovea. Nevertheless, despite its numerous propliopithecine characters, *Moeripithecus markgrafi* from Oman²² shares some characters with *Siamopithecus*. Both species show strong molar flaring, reduced and mesially displaced entoconid, weak hypoconulid, central lower molar constriction, and M^1 larger than M^2 with weak hypocone. Finally, the only synapomorphy observed in *Siamopithecus* and the Chinese *Eosimias* is the M_3 trigonid appreciably wider than the talonid. This situation can be explained by the strong difference of evolutionary grade between both taxa. *Siamopithecus* is a highly derived primate in comparison with *Eosimias*.

Siamopithecus therefore displays many anthropoid characters, which are nevertheless associated with some autapomorphic and primitive characters. The high number of anthropoid features indicates a low probability of independent evolution. On the other hand, as reflected by the cladistic analysis based on the available characters, *Siamopithecus* shares several derived features with the African propliopithecids (Fig. 2) which testify to its high evolved grade. *Siamopithecus* might be a representative of a branch issued from an African–Southeast Asian anthropoid radiation.

Godinot and Mahboubi⁷ have suggested very early divergence ages among anthropoid groups which makes such an evolutionary scenario coherent. In any case, this southeast Asian radiation seems to have been unexpectedly diversified, and *Siamopithecus* appears as a link allowing unification of *Pondaungia* and *Amphipithecus* as distinct lineages issued from a southeast Asian anthropoid, which may be *Eosimias*, when intermediate fossils are discovered, or an African migrant. Simons²³ recently stated that the Burmese forms could be emigrants from Africa or from anywhere else.

In this context, some groups of mammals strongly support South Asian–African migrations^{24,25}. The anthracotheriid genus *Bothriogenys* is known in Krabi (Thailand) and in the Oligocene Fayum deposits, and the species from both areas display affinities that suggest close relationships²⁵. The same is true for phiomysid rodents, which had a south Asian ancestor²⁶, and whose oldest representatives are known from the Late Eocene Nementcha locality in Algeria^{27,28}. Moreover, anomalurid and phiomysid rodents have been recently recognized in the Krabi mine from a level located some 40 metres above the main lignite seam (out unpublished data). In addition, the occurrence of anomalurid and phiomysid rodents along with *Moeripithecus* in both Egypt and Oman²⁹ supports the likelihood of mammal exchanges between North Africa and eastern provinces. The ancestor of the southeast Asian anthropoids therefore may have also originated in Africa, and then migrated into Southeast Asia during or after the Early Eocene⁷, but before the end of the middle Eocene. This ancestor, either African or Asian, may have undergone a local radiation and evolution there, and probably gave rise to several lineages, among which those of *Amphipithecus*, *Pondaungia* and *Siamopithecus*, taxa so far restricted to Southeast Asia.

In any case, Southeast Asia has definitely played a key role in the early evolution of anthropoids. □

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Language-specific phoneme representations revealed by electric and magnetic brain responses

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There is considerable debate about whether the early processing of sounds depends on whether they form part of speech. Proponents of such speech specificity postulate the existence of language-dependent memory traces, which are activated in the processing of speech^{1–3} but not when equally complex, acoustic non-speech stimuli are processed. Here we report the existence of these traces in the human brain. We presented to Finnish subjects the Finnish phoneme prototype /e/ as the frequent stimulus, and other Finnish phoneme prototypes or a non-prototype (the Estonian prototype /õ/) as the infrequent stimulus. We found that the brain's automatic change-detection response, reflected electrically as the mismatch negativity (MMN)^{4–10}, was enhanced when the infrequent, deviant stimulus was a prototype (the Finnish /õ/) relative to when it was a non-prototype (the Estonian /õ/). These phonemic traces, revealed by MMN, are language-specific, as /õ/ caused enhancement of MMN in Estonians. Whole-head magnetic recordings^{11,12} located the source of this native-language, phoneme-related response enhancement, and thus the language-specific memory traces, in the auditory cortex of the left hemisphere.

Our subjects were Finns and Estonians, who speak closely related languages with very similar vowel structures^{13–15} except that only Estonians have the vowel /õ/, which is roughly between /ö/ (as in 'sir') and /o/ (as in 'sore') (Fig. 1a). To determine the phonemes best representing the vowel prototypes /e/ (as in 'net' but longer in duration), /ö/ and /o/ (and also /õ/ for Estonians), we asked subjects to categorize phoneme stimuli varying in the second-formant (F2) frequency while the F1, F3, and F4 frequencies as well as the fundamental frequency were kept constant. Finns and Estonians judged the vowels common to the two languages similarly (Fig. 1b). In addition, the /õ/ category could also be observed in the responses of Estonians (Fig. 1b). Finns had a gap in this position of their response profile, suggesting that they could perceive the stimuli giving rise to the prototype /õ/ perception in Estonians as different from the prototype phonemes of their own language. Thus, although perhaps forming a perceptual category, the Finns did not consider this foreign phoneme prototypical, because they had not experienced it in their own language.

We then presented to 13 normal-hearing Finnish subjects (aged 18–29 years, right-handed, 4 females and 9 males) the prototype /e/ as the standard stimulus, randomly replaced by infrequent deviant stimuli that differed from the standard stimulus only in F2 (Fig. 1a). In further stimulus blocks, simple sinusoidal tones with frequencies

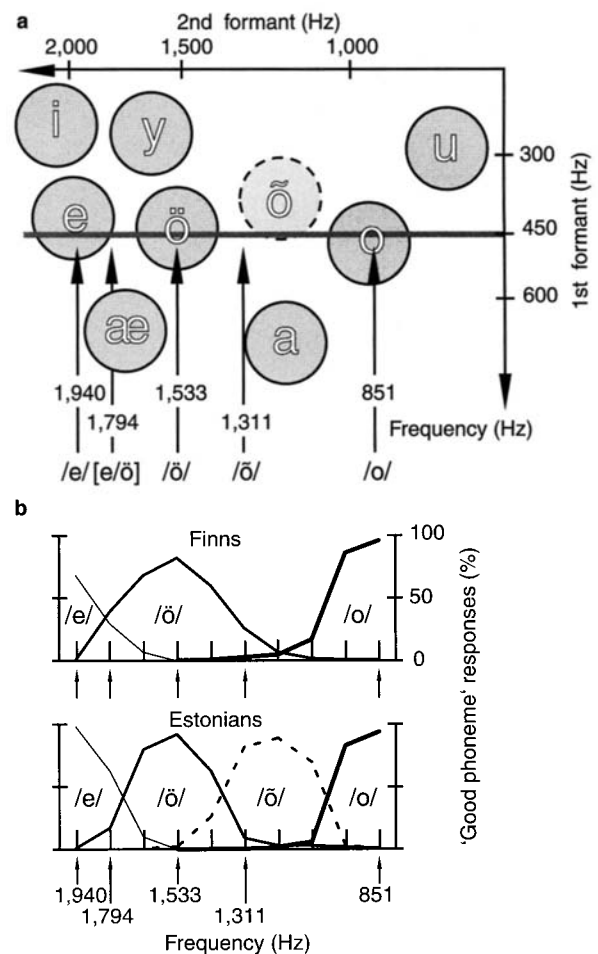


Figure 1 a. The arrows show locations of the vowel stimuli in the F1–F2 space. The standard stimulus was the Finnish and Estonian prototype /e/; the deviant stimulus [e/õ] was an intermediate between /e/ and /õ/; the deviant stimulus /õ/ was a prototype shared by the two languages; the deviant stimulus /ö/ was an intermediate between the Finnish and Estonian prototypes /ö/ and /o/ (and closely corresponded to the Estonian prototype /õ/); the deviant stimulus /o/ was a prototype in both languages. **b.** The percentages of 'good phoneme' /e/, /ö/ and /o/ responses in Finns (top) and of 'good phoneme' /e/, /ö/ and /o/ responses in Estonians (bottom) in the behavioural task. The ten stimuli used in the judgement task are indicated with tick marks. The frequency scales are logarithmic.

equal to these F2 frequencies were used in an otherwise identical paradigm. Brain electric responses to these stimuli were recorded while subjects were reading self-chosen text under the instruction to ignore all auditory stimuli.

Responses to the simple tone and its frequency changes are shown in Fig. 2. The standard stimuli elicited a P1–N1–P2 waveform, while the deviant-stimulus response was dominated by the MMN, an auditory response generated by an attention-independent change-detection process⁵. As expected, the MMN amplitude increased as a function of the frequency deviance⁷ (Fig. 2). That the present MMN to phonemes occurred independently of attention was supported by the absence of any subsequent slow positivity^{6,7} (P3 or P3a; Fig. 3).

Brain responses to phonemes are presented in Fig. 3a. Consistent with results indicating that MMN is enhanced with increasing frequency deviation⁷ (Fig. 2), the MMN amplitude was, in general, larger with greater F2 deviation from the standard stimulus /e/ (Figs 3a and 4a). In striking contrast, the deviant /õ/ (prototype) elicited a larger MMN than did the deviant /ö/ (non-prototype), although /õ/

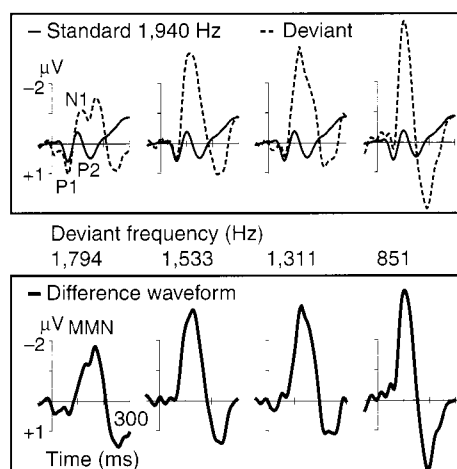


Figure 2 Top, the electric response (from the frontal Fz electrode; grand-average waveforms of Finnish subjects) to the standard sinusoidal tone of 1,940 Hz (solid line; equal to the F2 of /e/ used as the standard stimulus in the present phoneme experiments) and to deviant sinusoidal tones of 1,794, 1,533, 1,311 and 851 Hz (broken line; equal to the F2 of the deviant stimuli [e/o], /ö/, /õ/, and /o/, respectively). The standard-stimulus response shows relatively small P1, N1 and P2 deflections⁶. The negative displacement of the deviant-stimulus response relative to the standard-stimulus response is caused by the mismatch negativity (MMN), which can be seen by subtracting the standard-stimulus trace from that for the deviant stimulus (bottom). MMN steadily increases in amplitude with the increasing magnitude of frequency change.

deviated acoustically less from /e/ than did /õ/. Thus MMN was enhanced in size for prototype deviants from that attributable to the mere acoustic difference. In contrast to the MMN amplitude, the MMN latency was an orderly function of the magnitude of acoustic deviation, with the prototypicality of the deviant stimulus having no effect (Fig. 4b).

The deviant /õ/, although not a phoneme in the Finnish language, is a phoneme in Estonian. Thus, in Estonians, the MMN amplitude should not drop from deviant /õ/ to deviant /ö/. To verify this, 11 normal-hearing Estonian subjects (19–31 years old, right-handed, 6 females and 5 males) were subjected to the same experimental paradigm as the Finns. The drop in MMN amplitude from /õ/ to /ö/ seen in Finns did not occur in Estonians (Figs 3b and 4a), which must be due to /õ/ being a prototype in Estonian but not in Finnish. A two-way ANOVA (language versus deviants /õ/ and /ö/) showed a significant interaction term ($F(1, 22) = 11.16$, $P < 0.005$), indicating that the MMN amplitude as a function of deviance was different in Estonians and Finns. This language-specific prototypicality effect suggests the existence of neural traces of language-specific phoneme representations.

Magnetic responses to the deviant stimuli (MMNM, the magnetic equivalent of the electric MMN) were recorded from 9 of the Finnish subjects^{9,11,16}. The pattern of responses over the left hemisphere was the same (the diminished /õ/ response) (Fig. 4c, d) as that shown by the electric responses (Fig. 4a). Furthermore, the magnetoencephalogram showed that MMNM was larger in the left than in the right hemisphere when the deviant stimulus was a prototype (left, 34 fT cm^{-1} ; right, 22 fT cm^{-1} ; ANOVA, laterality $\times$ prototypicality interaction, $F(1, 8) = 6.27$, $P < 0.05$; see Fig. 4d, top). The equivalent current dipole^{11,12} of the left-hemisphere response showed that MMNM originated at the left auditory cortex and, further, that its dipole moment (strength) was considerably greater for all prototype deviants than for the non-prototype deviant /õ/ (see Fig. 4c, d). The right-hemisphere responses were not strong enough for us to determine the corresponding equivalent current dipole for each subject. As

was the case with the electric MMN, the MMNM latency steadily decreased with increasing acoustic deviation, there being no prototypicality effect.

Thus a vowel prototype of the subject's native language presented as the deviant stimulus elicited a considerably larger MMN (MMNM) than could have been expected on the basis of the acoustic (F2) distance from the frequent, standard phoneme alone. That is, the MMN amplitude was enhanced when the deviant stimulus was a prototype as opposed to an equally complex non-prototype. Furthermore, this MMN-amplitude enhancement pattern reflected the native-language phonology: when the deviant stimulus was a phoneme prototype in Estonian, but not in Finnish, the enhancement occurred in Estonian subjects but not in Finns. We therefore suggest that, in principle, our MMN paradigm could help to unravel the experience-dependent neural memory traces for phonemes of any given language.

We found cortical memory traces of speech sounds. These traces probably serve as recognition patterns¹⁷ for speech sounds, and allow the correct perception of a given language: they are activated by corresponding (and roughly corresponding, the category effect) speech sounds. These recognition patterns presumably develop gradually with exposure to the language; behavioural data suggest they develop within the first year of life¹⁸.

The left-hemisphere dominance of the MMNM enhancement to phoneme prototypes (Fig. 4c, d) suggests that the neural phoneme traces are located in the left auditory cortex. That the locus of the memory trace is indeed indicated by the locus of MMN (MMNM) generation is supported by the feature-specific loci of MMN generation in the auditory cortex^{19–21}. In contrast to the left-hemisphere results, in the right auditory cortex both prototypes and non-prototypes elicited a small MMNM of a similar size that resembles the response elicited by non-prototypes in the left auditory cortex. Thus the magnetic results indicate that the left auditory cortex is involved in phonemic discrimination, presumably because the neural phoneme traces are located there, whereas both the left and right auditory cortices serve acoustic discrimination. □

Methods

Stimuli. The F2 frequency for the semi-synthetic standard stimulus was chosen so that it produced the best exemplar representing /e/ (prototype), as judged by the subjects when the F2 frequency was changed in 4% steps. The first (F1), third (F3) and fourth (F4) formants were constant at 450, 2,540 and 3,500 Hz, respectively. The average fundamental frequency (F0) of each semisynthetic vowel was 105 Hz. Vowels were generated by the production of synthetic stimuli from natural glottal excitation in conjunction with a vocal-tract model^{22,23}. This method is based on a speech-production model assuming that speech is produced as a cascade of three independent processes: the glottal excitation,

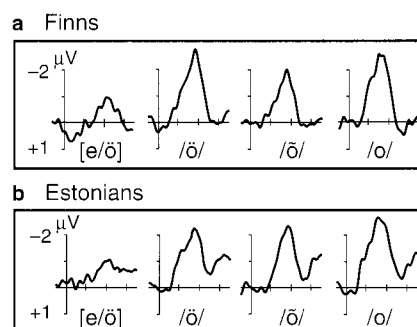


Figure 3 The amplitude of the mismatch negativity (MMN; frontal Fz electrode; grand-average deviant-standard difference waveforms) reflects the language-specific phoneme categories of the Finnish and Estonian languages. Note that the MMN amplitude for the deviant /õ/, a non-prototype in Finnish, is clearly smaller than that for the adjacent prototype deviants with Finnish subjects (a) but not with Estonian subjects (b) for whom /õ/ was a prototype.

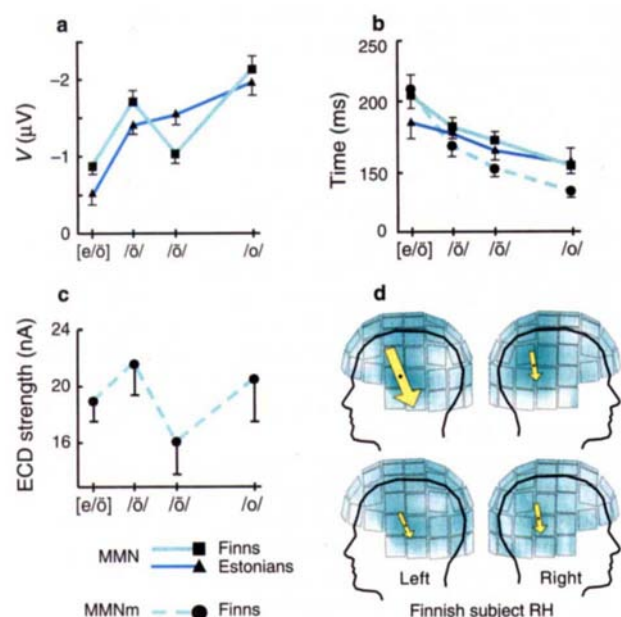


Figure 4 **a**, MMN peak amplitude (at Fz) in Finns (blue) and Estonians (purple) as a function of the deviant stimulus, arranged in the order of increasing F2 difference from the standard stimulus. Bars indicate s.e.m. **b**, MMN (at Fz) (solid lines) and MMNM (left hemisphere) (broken line) peak latencies as a function of the deviant stimulus for Finns (blue) and Estonians (purple). **c**, Strength of the equivalent current dipole (ECD; average of 9 Finnish subjects) modelling the left auditory-cortex MMNM for the different deviant stimuli. **d**, Left- and right-hemisphere MMNMs of one typical Finnish subject for deviants /ö/ and /ä/ presented in contour (spacing 2 fT cm⁻¹) maps of the magnetic field-gradient amplitude at the MMNM peak latency. The squares indicate the arrangement of the magnetic sensors. The arrows represent ECDs indicating activity in the auditory cortex; the black dots in these arrows show the centres of gravity of MMNM. Note that the prototype /ö/ elicits a much larger MMNM in the left than in the right hemisphere, whereas non-prototype /ä/ responses in both hemispheres are small and quite similar in amplitude.

the vocal tract, and the lip-radiation effect²². In this way we could accurately adjust the formants of the stimuli without making sound quality too artificial. The excitation process was computed from a male voice (vowel /a/, normal phonation) using an automatic inverse filtering technique²³. The vocal tract was modelled using an eighth-order all-pole filter with coefficients adjusted to shift the second formant. The lip-radiation effect was modelled with a fixed differentiator.

Behavioural task. The stimuli, presented at 1,400-ms onset-to-onset intervals, were 75 dB in intensity and 400 ms in duration (with rise and fall times of 10 ms each). Each stimulus was presented about 30 times. Finnish subjects were instructed to press the /e/, /ö/ or /o/ button when the stimulus sounded as a good representative of that category. For Estonians, there was also a response button for their /ä/.

Electric measurements. Measurements were performed in an acoustically and electrically shielded room. Blocks of the vowel /e/ were binaurally presented (75 dB, with a duration of 400 ms including 10-ms rise and fall times) through headphones as the standard stimuli, 15% of the stimuli were randomly appearing deviant sounds (one type per block). The onset-to-onset inter-stimulus interval was 900 ms. The nose-referenced electroencephalogram (EEG, sampling rate 250 Hz) was averaged and digitally filtered (passband 1–30 Hz). Epochs with artefacts exceeding 100 µV at any electrode or with an electro-oculogram (EOG) response exceeding 150 µV were discarded. The baseline for the waveforms was defined as the mean amplitude between -50 and 0 ms relative to stimulus onset. The MMN amplitude was determined from the Fz electrode (where it was usually largest) separately for each subject as the mean amplitude of the 150-ms period centred at the largest peak between 100 and 240 ms.

Magnetic measurements. The experimental paradigm was the same as for

the electric measurements. The magnetic responses were measured using a helmet-shaped, whole-head Neuromag-122 magnetometer²⁴ (sampling rate 397 Hz). The stimuli were delivered through plastic tubes and earpieces at 60 dB above the individual hearing level. Epochs with an electro-oculogram or magnetoencephalogram change exceeding 150 µV or 1,500 fT cm⁻¹, respectively, were omitted from subsequent analysis. The locus of the neuronal activity was estimated by determining the equivalent current dipole at the MMNM peak latency using 40–44 channels separately over the left and right frontotemporal cortices.

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Bcl-2 promotes regeneration of severed axons in mammalian CNS

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Most neurons of the mammalian central nervous system (CNS) lose the ability to regenerate severed axons *in vivo* after a certain point in development¹. At least part of this loss in regenerative potential is intrinsic to neurons^{2–4}. Although embryonic retinal ganglion cells (RGCs) can grow axons into tectum of any age, most RGCs from older animals fail to extend axons into CNS tissue derived from donors of any age, including the embryonic tectum². Here we report that the proto-oncogene *bcl-2* plays a key role in this developmental change by promoting the growth and regeneration of retinal axons. This effect does not seem to be an indirect consequence of its well-known anti-apoptotic activity. Another anti-apoptotic drug, ZVAD, supported neuronal survival but did not promote axon regeneration in culture. This finding could lead to new strategies for the treatment of injuries to the CNS.

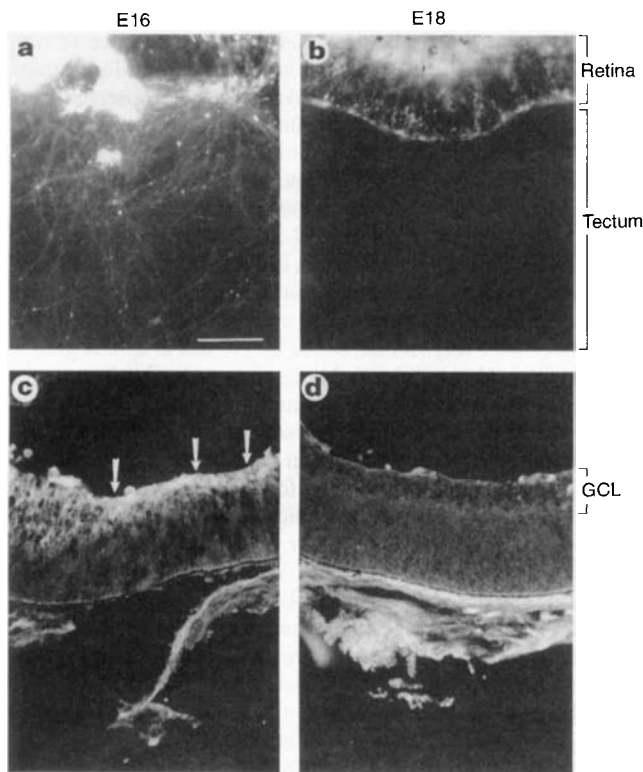


Figure 1 The level of expression of *bcl-2* in RGCs correlates with the growth ability of retinal axons. **a, b**, Epifluorescence photomicrographs of Dil-labelled retinal axons in cultures prepared from E16 (**a**) and E18 (**b**) wild-type mice. **c, d**, Immunofluorescent staining of Bcl-2 on transverse sections of retinae obtained from E16 (**c**) and E18 (**d**) embryos. Axons from E16 retinae (**a**) can be observed growing into the entire tectal explant; correspondingly, labelling of Bcl-2 appears positive in the retinal ganglion-cell layer (GCL) (**c**). However, the retinal explant derived from E18 animals (**b**) fails to grow axons into the tectal tissue, and the labelling of Bcl-2 disappears from the retinal ganglion-cell layer (**d**). Arrows in **c, d** point to the retinal ganglion cell layer with positive labelling. Scale bar, 200 µm.

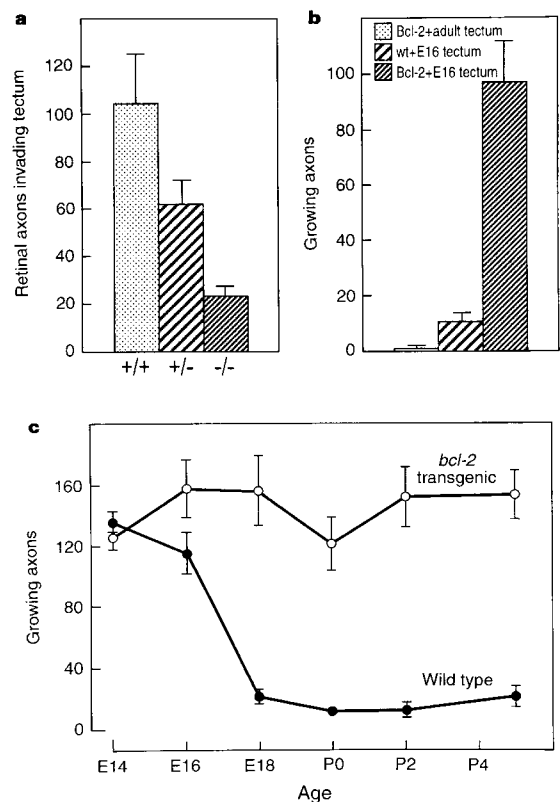


Figure 2 The expression of *bcl-2* is essential for the growth of most retinal axons in culture. Quantification of retinal axon growth observed in cultures from wild-type (C57BL/6J), *bcl-2* null and *bcl-2* transgenic mice. **a**, Quantification of cultures derived from E15 pups genetically deficient in *bcl-2*. Retinal explant derived from heterozygous (+/-) or homozygous (-/-) mutant mice both showed decreased numbers of axons that invaded the tectal tissue when compared with those of wild-type animals (+/+) at this age. **b**, Quantitative results for the growth of retinal axons from adult retinae. Retinal explants derived from adult transgenic mice display 10-fold more axonal growth into E16 tectum than seen from retinal explants from wild-type mice. **c**, Growth curves of retinal axons obtained from retinotectal co-cultures, using tissues from wild-type or transgenic animals aged E14 to P5. Note that at age E18 or older there is a marked decrease in numbers of retinal axons from wild-type animals. This decline was not observed for *bcl-2* transgenic mice.

To examine the growth of CNS axons of mice, we established an organotypic co-culture model of the retinotectal system in which the growth pattern of retinal axons closely mimics that seen *in vivo*². Tissues from retinae and midbrain tecta of C57BL/6J mice were abutted in a culture well. Quantitative analysis of axonal growth from retinae was achieved by standard placement of four Dil crystals into each retinal explant. We first examined co-cultures prepared from animals aged embryonic day 14 (E14, where E0 was the day of mating) to E16. Growth of retinal axons into the tectal slice was extensive ($n = 20$) (Figs 1 and 2). The number of labelled axons invading tectal tissue averaged 126 ± 10.0 . In contrast, retinal explants ($n = 60$) prepared from animals at age E18 and older exhibited markedly reduced axonal growth. For E18 tissue, the mean number in the labelled sample was 15.5 ± 3.3 fibres per tectal slice (Figs 1b and 2c). This indicated that, starting at E18 in mice, most RGC axons fail to regenerate in culture. These findings are similar to those previously reported on the Syrian hamster².

To examine whether the observed age-dependent reduction in the growth of retinal axons is due to increased RGC death, we counted the dying cells in retinal explants, using Sytox green fluorescent

dead-cell stain. There were 121 ± 21 cells mm^{-2} ($n = 4$) and 138 ± 19 cells mm^{-2} ($n = 3$) for E16 and E18 retinal explants, respectively, indicating that there was no significant increase in cell death before and after the regenerative failure occurred. We also counted the cells in the RGC layer, and found 85.8 ± 1.7 ($n = 3$) and 80.4 ± 2.1 ($n = 3$) cells per 200-µm retinal segment at E16 and E18, respectively. At either age, few pyknotic cells were found in the RGC layer. It has been shown that the prenatal ganglion cell layer comprises almost entirely RGCs⁵, so these data suggest that there is no significant change in RGC number between E16 and E18 retinae. Therefore, the observed age-dependent reduction in the growth of retinal axons cannot be attributed to the loss of RGCs.

We have previously shown that the regenerative failure of most of the older axons seems to be controlled by a genetic programme executed in developing neurons². To determine which genes might play such roles in regulating the growth of retinal axons, we compared the level of expression of several molecules, including neural cell adhesion molecule (NCAM), β -amyloid precursor protein and *bcl-2* protein (Bcl-2), with the use of immunofluorescence staining. We found high expression of *bcl-2* at E16 in

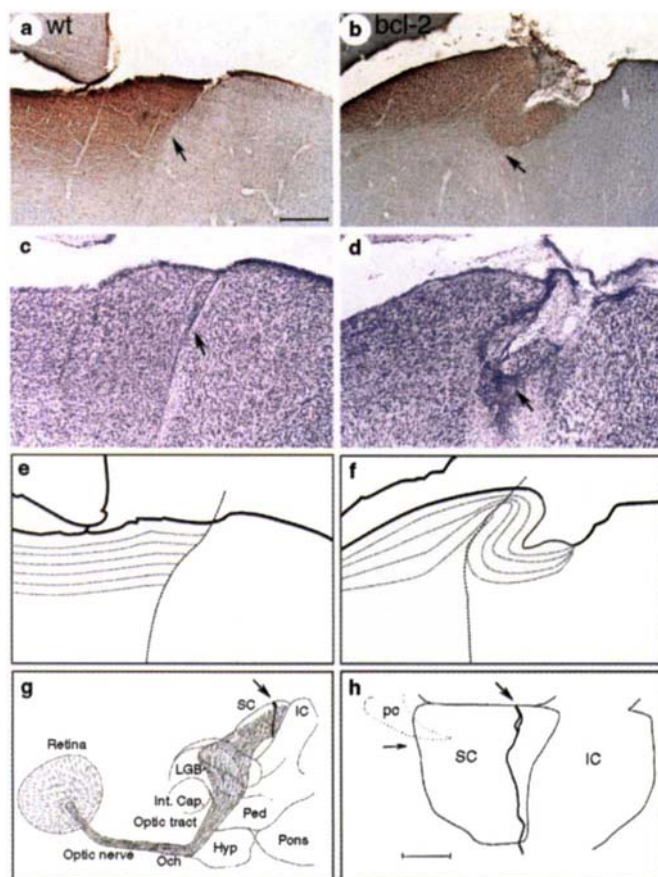


Figure 3 Expression of *bcl-2* in transgenic mice leads to regeneration of retinal axons after optic-tract transection *in vivo*. **a, b**, Photomicrographs of anterogradely labelled retinotectal axons after optic-tract transection; **c, d**, staining of adjacent brain sections with cresyl violet; **e, f**, drawings of **a-d**, using dotted lines to highlight the path of retinotectal projections and the bold dashed line for the lesion. **g**, A reconstructed view of the superior colliculus from one case. Note that in wild-type (wt) animals (**a**), labelled axons do not cross the lesion site; those from *bcl-2* transgenic mice (**b**) regenerate across the lesion site and enter the caudal tectum. Arrows point to the lesion site; additional arrows in **h** indicate the position of the sagittal sections (**b, d**). Abbreviations: Hyp, hypothalamus; IC, inferior colliculus; Int. Cap., internal capsule; LGB, lateral geniculate body; Och, optic chiasm; pc, posterior commissure; Ped, cerebral peduncle; SC, superior colliculus. Scale bar, 200 μ m.

the RGC layer ($n = 3$) (Fig. 1c); at E18, in parallel with the onset of regenerative failure in culture, the expression of *bcl-2* decreased to an undetectable level ($n = 3$) (Fig. 1d).

To determine whether *bcl-2* is required for the growth of retinal axons, we studied a loss-of-function animal model: mice genetically deficient in *bcl-2* (ref. 6). Co-cultures were prepared from E15 embryos that contained wild-type, heterozygous and *bcl-2*-deficient mice. At this stage, retinal explants of wild-type animals showed robust neurite outgrowth. However, in cultures prepared from heterozygous animals, the number of labelled retinal axons was reduced by about 50% (62 ± 8 , $n = 20$); in those from homozygous animals, the number was reduced by about 80% (22 ± 4 , $n = 7$) (Fig. 2a). To exclude the possibility that tectal tissues from mutant mice may affect axonal growth of RGCs, retinal explants from each animal were also co-cultured with the tectum from a wild-type, heterozygous or homozygous animal. There was no significant difference between groups of retinal explants co-cultured with tectal tissue from wild-type and mutant mice (data not shown). The number of

retinal axons from cultures of mice containing the homozygous *bcl-2* mutation was equivalent to that of wild-type mice at E18, when most RGCs failed to grow axons into tectum. To examine further whether the differences in axonal growth observed were accounted for by the difference in the number of neurons in the retinae, RGC numbers were assessed in the retinae of six mice: two wild-type, three *bcl-2* heterozygous, and one *bcl-2* homozygous. The mean numbers of RGCs counted in 200- μ m retinal segments in our sections were 89.3 ± 1.1 , 89.0 ± 0.7 and 87.1, respectively. The number of RGCs among 28 sections of each animal showed little variation (s.e.m., 3.5–6.5% of mean within each retina). The size of the eyeball (diameter, 0.83–0.85 mm) and the diameter of the RGCs of these mice were very similar, so the differences in axonal growth observed with wild-type mice and mice mutated for *bcl-2* cannot be accounted for by differences in the number of RGCs.

Because loss of Bcl-2 function represses axonal growth, we wished to determine whether overexpression of *bcl-2* in adult retinae is sufficient for retention of the ability for retinal axon regeneration. Therefore, we analysed mice transgenic for the *bcl-2* gene driven by the neuron-specific enolase promoter^{7,8}; our study was performed on line 73 of these transgenic mice. We examined co-cultures of retinae and tecta derived from animals aged E14 to postnatal day 5 (P5, where P0 is the day of birth), which covered the period before and after regenerative failure normally occurs. As previously described, retinal explants from each mouse were co-cultured with tecta of wild-type or transgenic mice. Starting at E18, retinal explants from wild-type mice exhibited a failure of RGC axon elongation ($n = 15$) (Fig. 2c), regardless of whether they were confronted with wild-type or transgenic tectal tissues. The number of labelled retinal axons decreased 10-fold in comparison to E16 retinal explants. In contrast, when retinae were derived from *bcl-2* transgenic animals, all retinal explants, collected from animals aged E14 to P5, showed extensive fibre outgrowth ($n = 35$) (Fig. 2c). No difference was observed in the number of retinal axons that invaded tectal slices derived from wild-type and *bcl-2* transgenic mice (data not shown). Furthermore, it has been reported that, in the retinae of the transgenic mouse line 73, *bcl-2* is detected primarily in RGCs⁷. We therefore conclude that constitutive expression of *bcl-2* in RGCs, rather than in the CNS environment of the axon, overcomes regenerative failure of retinal axons in the perinatal period.

RGCs derived from *bcl-2* transgenic mice retained the ability to grow axons throughout their lifespan. Extensive neurite outgrowth was observed from adult retinal explants of transgenic mice when they were co-cultured with E16 tectal slices ($n = 10$); the number of labelled retinal axons averaged 96.3 ± 15.3 , almost equivalent to the number obtained from an E16 retinotectal co-culture. However, when the adult retinae were confronted with adult tectal tissues, little axonal growth was achieved ($n = 13$) (Fig. 2b). We do not yet know why adult tectal tissue prevented axon regeneration from retinae of mice overexpressing *bcl-2*. It is likely that adult CNS contains inhibitory molecules, such as myelin-associated inhibitory proteins, that are absent in embryonic tissues⁹ or that they express low levels of neurotrophic factors that are required for the regrowth of retinal axons. Despite these factors, the normal embryonic retinae can grow axons into adult tectum², indicating that important factors additional to *bcl-2* are probably involved. In any case, we can conclude that *bcl-2* is central to the regulation of the intrinsic genetic program for retinal axonal growth, and that *bcl-2* is essential, but not sufficient, for the regeneration of retinal axons in mature CNS.

We then investigated regeneration of retinal axons *in vivo*. Young pups (P4) obtained from the mating of males heterozygous for the *bcl-2* transgene and C57BL/6J females received a unilateral transection of the optic tract at the mid-tectal level (Fig. 3c, d, g, h). Axonal regrowth was assessed by tracing the retinal projection fibres with cholera toxin B-subunit (CT-B)¹⁰. To visualize the lesion site,

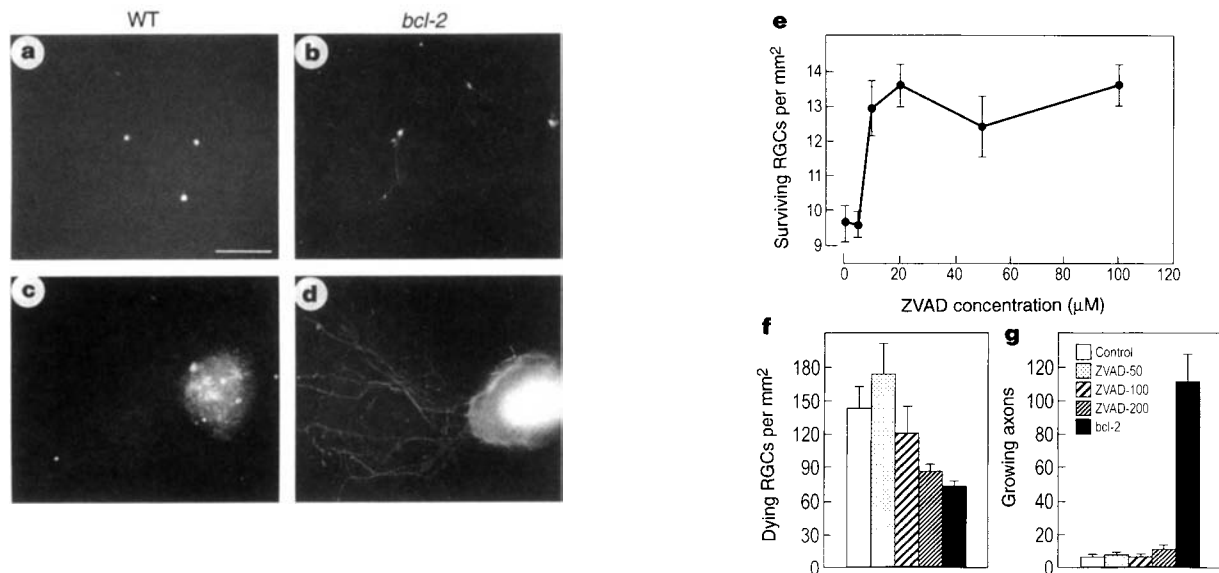


Figure 4 ZVAD, although sufficient to prevent the death of RGCs, is not sufficient to promote axonal growth. **a–d**, Epifluorescence photomicrographs of Dil-labelled RGCs obtained from dissociated (**a, b**) and explant (**c, d**) retinal cultures using tissues from P2 wild-type (**a, c**) or transgenic (**b, d**) mice. RGCs from wild-type mice (wt) surviving in ZVAD-treated cultures are round and devoid of neurites. In contrast, RGCs from transgenic mice (*bcl-2*) extend long axons in culture. **e**, Surviving RGCs in dissociated retinal cell cultures treated with different doses of ZVAD; doses from 0 to 200 μM were tested. **f**, Quantification of cell death

in retinal explants from ZVAD-treated retinotectal co-cultures: three doses of ZVAD (50, 100 and 200 M) were examined, and cultures were prepared from P2 wild-type animals. **g**, Quantification of retinal axon growth in co-culture experiments parallel to those in **f**. By increasing the concentration of ZVAD, the number of dying cells in retinal explants decreased, but the number of growing axons did not change significantly. All data represent at least 3 replicated experiments. Scale bar, 200 μm.

alternate sagittal sections of these brains were collected for cresyl violet staining and reconstructed in three-dimensions with the Neurotrace program. In wild-type mice, the retinotectal projection was restricted to the tissue proximal to the lesion site ($n = 5$) (Fig. 4a, c, e). In contrast, axotomized retinal axons in transgenic mice grew in large numbers across the lesion site and innervated the tectum caudal to the injury ($n = 6$) (Fig. 3b, d, f). Remarkably, in three transgenic mice, the lesion produced a large, impassable gap in the superficial superior colliculus, but the axons were observed to go around the gap, and then pass straight through the lesion site below the gap on their way to the target tissue (Fig. 3b, f), without the addition of any bridging material or neurotrophic factors. Regenerating axons reached the posterior border of the superior colliculus. Our three-dimensional reconstruction showed that axons innervated the entire area of superior colliculus caudal to the lesion site. No axons were observed to invade the inferior colliculus. Although it has been documented that larger numbers of neurons and axons were found in these mutants than those in wild-type animals^{7,8}, the retinofugal projections to the lateral geniculate body and superior colliculus of transgenic mice seemed to be very similar to those in normal wild-type mice (data not shown). These results demonstrate that *bcl-2* promotes retinal axon regeneration *in vivo*.

It should be emphasized that the above experiment was designed to result in a minimal amount of tissue damage, so that large numbers of RGCs in wild-type mice survived after injury; however, they were unable to regenerate severed axons. Similar observations suggesting a dissociation of neuronal survival and axonal regrowth after axotomy have previously been reported¹¹.

The anti-apoptotic function of Bcl-2 is well established^{12,13}, so it is important to establish whether its growth-promoting activity is simply an indirect consequence of increased cell survival. It has been suggested that Bcl-2 suppresses apoptosis by impairing the activity of interleukin-1 β -converting enzyme (ICE)^{14–17}, a cysteine protease thought to be essential in the apoptotic process in vertebrates^{14–16}. To test the relationship between the functions of axonal growth and cell survival, we added the irreversible ICE-like protease inhibitor

ZVAD (Z-Val-Ala-Asp-CH₂F; Enzyme Systems Products)^{16,18} in a dissociated cell culture system that allows visualization of single cell morphology. We prepared cultures from retinotectal P2 pups, and RGCs were prelabeled by injecting Dil into the tectum of P0 pups. We plated 10⁵ cells that contained 4.8% (averaging 4,800) RGCs in each well of 24-well plates. Treatment with ZVAD at a concentration of 10 μM or above effectively reduced RGC death after 2 days in culture. The number of surviving RGCs was increased from 25% (without ZVAD) to about 40% (Fig. 4e). With this concentration of ZVAD, cultures derived from wild-type and *bcl-2* transgenic mice contained equal numbers of surviving RGCs (data not shown). Nevertheless, labelled RGCs from wild-type animals were round and devoid of neurites in culture ($n = 36$ cultures) (Fig. 4a, c), whereas RGCs derived from *bcl-2* transgenic mice ($n = 24$ cultures) exhibited extensive axonal outgrowth (Fig. 4b, d). Note that this occurs in the absence of any neurotrophic factors added to the culture medium. To test the possibility that wild-type RGCs do not grow neurites because of a shortage of neurotrophic factors, we added BDNF and NT-3 to the culture medium. These factors increased the number of surviving RGCs, as they had *in vivo*¹⁹, but the proportion of RGCs with regenerating axons among the total number of surviving RGCs remained unchanged from that of the untreated control (data not shown).

We also tested the effect of the ICE inhibitor in our explant co-culture system with tissue prepared from wild-type P2 mice. Treatment with ZVAD reduced the extent of cell death in retinal explants (Fig. 4f). As we found in dissociated retinal cultures, 200 μM ZVAD protected cells from death almost as well as did *bcl-2* in the transgenic mouse ($n = 6$) (Fig. 4f); however, the number of axons that invaded tectal slices was 10-fold less in cultures from wild-type animals than in those from *bcl-2* transgenic mice (Fig. 4g). Therefore, these experiments indicated that cell survival and axonal growth are two distinct activities of RGCs; Bcl-2, but not ICE inhibitors, supports both of these activities.

The dissociation between cell survival and axonal growth^{20,21} is supported by our observations. First, the regenerative failure of

retinal axons and the decrease in Bcl-2 levels in RGCs occur earlier (at E18) than most programmed cell death (P1–P5)²². This corresponds with the observation that the expression pattern of *bcl-2* does not mirror recognized patterns of cell death in the CNS²³; rather, it seems to correlate with cell differentiation and capacity for axonal outgrowth of neurons²³. Second, before birth the densities of RGCs are similar in retinal explants from wild-type mice and mice transgenic or with null mutations for *bcl-2* (refs 7, 8, 24), yet these neurons exhibited drastic differences in the ability to grow axons in culture. This implies that the differences in axonal growth observed in culture were not accounted for by differences in the number of RGCs in their retinæ. Finally, our experiments using ZVAD further demonstrated that ICE inhibitor, although sufficient to block cell death, is not sufficient to support axonal growth. These findings all suggest that Bcl-2 promotes axonal growth through a mechanism independent of its anti-apoptotic activity. However, we cannot exclude the possibility that, in *bcl-2* transgenic mice, a subpopulation of RGCs that possesses the ability to regrow its axons might be specifically rescued from death during development. The hypothesis of the growth-promoting function of Bcl-2 is supported by our findings, but also needs to be further tested.

It has recently been shown that Bcl-2 promotes neurite sprouting by regulating neural differentiation *in vitro*²⁵. It interacts with a member of the Ras family, R-ras p23 (ref. 26), and is co-immunoprecipitated with the serine/threonine-specific protein kinase Raf-1 (ref. 27). These molecules have been implicated as components of growth-factor-mediated signal-transduction pathways that regulate neurite outgrowth²⁸. Whether Bcl-2 acts through these molecules to execute its growth-promoting activity should be examined.

In summary, our results indicate that Bcl-2 can act as a regulatory switch for a genetic programme that controls the growth of CNS axons. This finding strengthens the notion that, for most developing CNS neurons, the ability to extend axons is governed by an intrinsic developmental programme. It provides a basis for the design of new therapeutic strategies for treatment of brain and spinal injuries, as well as many neurodegenerative diseases. □

Methods

Retinotectal co-culture. Retinotectal co-cultures were prepared essentially as described². Briefly, brains were dissected, and coronal slices through the superior colliculus were cut with a McIlwain tissue chipper at a thickness of 300 µm. Each retina was cut in half and abutted against a tectal slice. Tissues were placed on the microporous membrane of Millicell wells and maintained in NeuralBasal medium supplemented with B27 at 37 °C for 5 days. To exclude the possibility that tectal tissues from mutant mice may affect axonal growth of RGCs, we set up a series of parallel experiments in which one retinal explant of each mouse was confronted with the tectum from the same mouse, while the second retinal explant was placed against the tectum from another mouse. With this arrangement, retinal explants from each animal had the possibility of being co-cultured with the tectum from a *bcl-2* transgenic, wild-type, heterozygous or homozygous animal. Because mouse tails were collected after the animals had been killed for culturing, all the results were actually obtained and scored blind before the genotype was determined. The number of regenerating axons was sampled by applying four DiI crystals to each fixed retinal explant. Thus a small portion of RGCs in each explant was labelled. Data from each group were drawn from at least 6 explant cultures (equivalent to 24 samples). The co-cultures were stored in fixative for 2–4 weeks to allow diffusion of the dye, and labelled retinal axons were viewed with a fluorescence microscope (Nikon).

Quantification of RGC densities in retinæ. The eyecups from E16 and E18 mice were dissected and sectioned at 10 µm on a cryostat. The total number of sections was recorded to measure the diameter of the eyeball. The sections were then stained with cresyl violet. For each eye, three sections, including one with the optic nerve head and two others that were 300 µm away from the centre section, were selected. Cells contained in the RGC layer were counted in 6–11 fields (200 µm long) to cover the full length of each section. Pyknotic cells were identified by the presence of condensed and darkly stained nuclei⁵. Results are presented as mean ± s.e.m.

Immunofluorescence staining. E16 or E18 mouse embryos were obtained by Caesarian section of timed-mated, wild-type mothers. Brains were removed and fixed in 4% paraformaldehyde overnight and cut into transverse sections 10 µm thick with a cryostat. Sections were blocked with PBS containing 2.5% normal goat serum, 2.5% fetal bovine albumin, and 0.3% Triton X-100 for 30 min at room temperature, and then incubated with affinity-purified primary antibody (hamster anti-mouse *bcl-2*, 1:50; PharMingen) at 4 °C overnight. Secondary antibody (FITC-conjugated goat antibody to hamster immunoglobulin, 1:200) was then applied to the slide for 2 h at room temperature. The slides were rinsed several times in PBS, mounted in Fluoremount G, and viewed with the fluorescence microscope.

Surgery. Mouse pups were obtained from matings of males heterozygous for the *bcl-2* transgene with C57BL/6J females. Four days after birth (P4), pups received a unilateral transection of the optic tract at the mid-tectal level. To be sure the axons had been cut, transection was made with a knife followed by transverse passage of a tungsten hook that was pulled deeply through the superficial layers of superior colliculus, reaching the surface of the central grey. Regeneration of the optic tract was assessed using anterograde tracing with CT-B 10 days after the transection. To visualize the axons, a DAB reaction was performed using a slightly modified version of a previous protocol¹⁰. Briefly, brains were cut into 50-µm sagittal sections; alternate sections were collected for cresyl violet staining, and remaining sections were incubated with primary antibody against CT-B at 4 °C for 96 h and then further processed with ABC elite kit (Vector). Sections were visualized with a Nikon microscope. Serial sections from one transgenic mouse were used for a three-dimensional reconstruction with MIT Neurotrace computer software to obtain a detailed assessment of the width and depth of the lesion, and of the size of the reinnervated superior colliculus. Mouse tails were scored blind before the genotype was determined.

Dissociated retinal cell cultures. Primary cultures of dissociated retinal cells were prepared from P2 wild-type or transgenic animals. RGCs were prelabelled by injecting DiI solution (25% in dimethyl formamide) into the tectal region bilaterally in P0 pups. Cells were plated in 24-cell wells treated with poly-L-lysine (10 µg ml⁻¹ at 4 °C overnight) and coated with human merosin (0.2 µg ml⁻¹ at room temperature for 2 h)²⁹. We plated 10⁵ cells that contained 4.8% (averaging 4,800) RGCs in each well. The numbers of retinal cells and RGCs were counted before plating, using a Nikon microscope equipped with phase-contrast and fluorescence illumination. RGCs were identified by DiI labelling, and were found to comprise, on average, 4.8% of retinal cells at this age. Cultures were maintained for 2 days in NeuralBasal medium supplemented with B27, and the number of surviving RGCs were counted after culturing. Trypan blue staining was used to examine the viability of RGCs³⁴. Morphologically, cells undergoing apoptosis were distinguished by condensed chromatin and shrunken cell bodies³⁰. Retinotectal co-cultures prepared from wild-type P2 mice have been described² and ZVAD was added to the culture medium at the time of plating. Cell death was detected by staining with the fluorescent dye Sytox green fluorescent dead-cell stain (Molecular Probes).

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Competitive binding of α -actinin and calmodulin to the NMDA receptor

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The mechanisms by which neurotransmitter receptors are immobilized at postsynaptic sites in neurons are largely unknown. The activity of NMDA (N-methyl-D-aspartate) receptors is mechano-sensitive¹ and dependent on the integrity of actin², suggesting a functionally important interaction between NMDA receptors and the postsynaptic cytoskeleton. α -Actinin-2, a member of the

spectrin/dystrophin family of actin-binding proteins, is identified here as a brain postsynaptic density protein that colocalizes in dendritic spines with NMDA receptors and the putative NMDA receptor-clustering molecule PSD-95. α -Actinin-2 binds by its central rod domain to the cytoplasmic tail of both NR1 and NR2B subunits of the NMDA receptor, and can be immunoprecipitated with NMDA receptors and PSD-95 from rat brain. Intriguingly, NR1- α -actinin binding is directly antagonized by Ca^{2+} /calmodulin. Thus α -actinin may play a role in both the localization of NMDA receptors and their modulation by Ca^{2+} .

NMDA receptors exist *in vivo* as heteromultimeric complexes consisting of the essential NR1 subunit coassembled with various subunits of the NR2 subfamily^{3–8}. An association between NR2 subunits and the PSD-95/SAP90 family of synaptic proteins has been identified^{9,10}, but the nature of specific proteins that mediate NMDA receptor interaction with the postsynaptic actin cytoskeleton is not known. The carboxy-terminal intracellular domain of NR1 (Fig. 1) represents a potential target for intracellular anchoring molecules, especially as alternative splicing within this C-terminal region can affect the subcellular distribution of NR1 (ref. 11).

A yeast two-hybrid screen of a human brain complementary DNA library was performed using as bait the C-terminal tail from the most abundant NR1 splice variant (NR1A)¹² (Fig. 1a, b). This C-terminal splice variant contains both C-terminal exon cassettes (termed C1 and C2) as well as the membrane-proximal region (here termed C0), and is thus referred to as C0-C1-C2 (Fig. 1, inset). The two-hybrid screen yielded multiple isolates of two distinct clones (A1.7 and A2.10) which interacted specifically with the C-terminal intracellular tail of NR1, but not with Shaker-type K^+ channel subunits or the AMPA-receptor subunit GluR1. Clone A1.7 interacted with the C0-C1-C2 but not with the C0-C2 C-terminal variant and encoded a new protein with weak homology to muscle myosin heavy chain (M.W. and J.L., unpublished observations). Clone A2.10 interacted with both the C0-C1-C2 and C0-C2 variants of NR1 and contained a 2.2-kilobase (kb) cDNA encoding the C-terminal two thirds of the human α -actinin-2 gene (Fig. 1a)¹³. The

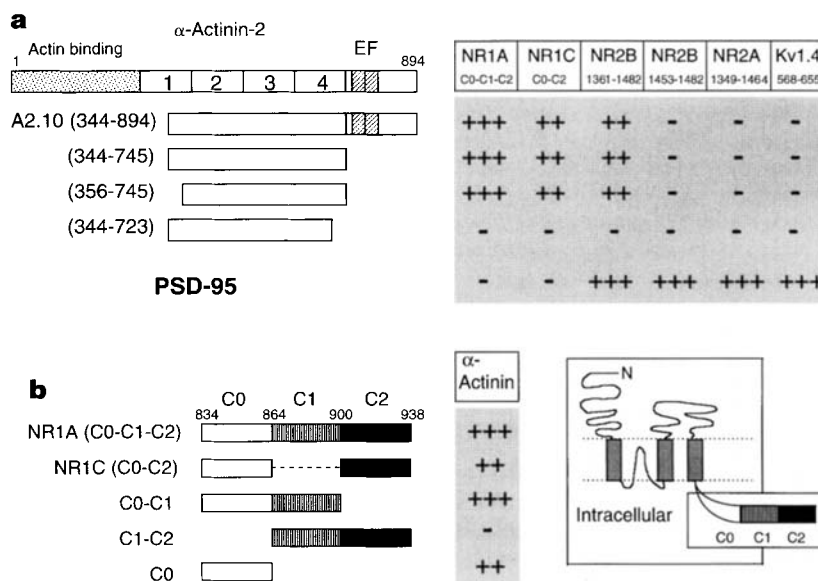


Figure 1 a, α -actinin-2 binds to the cytoplasmic tails of NR1 and NR2B through its central rod domain. Clone A2.10, and further deletion variants, are shown aligned under full-length α -actinin-2, in which the actin-binding domain, repeats 1–4 of the central rod domain, and EF hands are indicated. The interaction of α -actinin-2 constructs with the cytoplasmic tails of NR1, NR2B, NR2A and Kv1.4 are shown, as measured by the yeast two-hybrid assay. Numbers refer to amino-acid residues at the boundaries of each construct. Yeast two-hybrid interactions were semi-

quantified on the basis of induction of the reporter genes *HIS3* and β -galactosidase²¹. **b**, The C0 segment of the NR1 C-terminal tail is necessary and sufficient for binding to α -actinin-2. Deletion constructs of the NR1 C-terminal tail were tested for interaction with α -actinin-2 (A2.10) by yeast two-hybrid assay. Numbers refer to the amino-acid residues at the boundaries of C0, C1 and C2 segments²⁵. Inset, membrane topology of NR1. The intracellular tail consists of the membrane proximal segment C0, and C-terminal exon-segments C1 and C2.

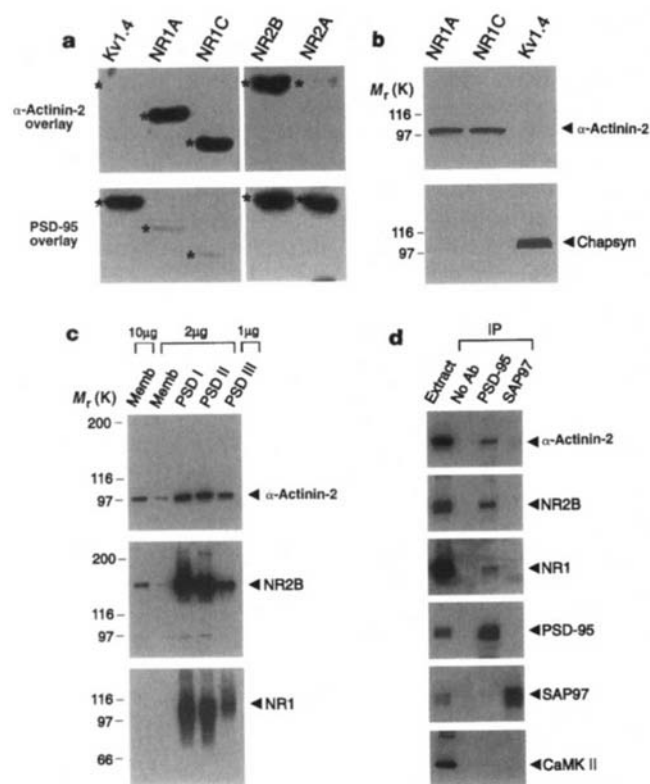


Figure 2 Biochemical association of α -actinin-2 and NMDA receptor subunits *in vitro* and in rat brain. **a**, Filter overlay assay showing specific *in vitro* binding of α -actinin-2 to NR1A, NR1C or NR2B C-terminal tails. GST-fusion proteins of the C-terminal tails of NR1A, NR1C NR2A, NR2B or Kv1.4¹⁰ were probed with a hexahistidine-tagged fusion protein of α -actinin-2 (top) or PSD-95 (bottom)²¹. Asterisks indicate the positions of the fusion proteins. **b**, The C-terminal tail of NR1 selectively 'pulls-down' α -actinin-2 from rat brain extracts. Detergent extracts of rat brain were incubated with GST-fusion proteins of the C termini of NR1A, NR1C or Kv1.4 coupled to glutathione-Sepharose beads. Bound proteins were immunoblotted for α -actinin-2 and chapsyn-110 (ref. 17). Positions of molecular size markers are shown. **c**, Copurification of α -actinin-2 and NMDA receptor subunits into the postsynaptic density (PSD) fraction. Lanes were loaded with rat brain fractions (10, 2 or 1 μ g, as indicated): crude synaptosomal membrane fraction (Membr); purified postsynaptic density fractions extracted with Triton X-100 once (PSD I), twice (PSD II), or with Triton X-100 followed by Sarkosyl (PSD III)²⁶. Immunoblots were probed for α -actinin-2, NR1 and NR2B (a major component of the PSD²⁷). **d**, Coimmunoprecipitation of α -actinin-2 with NMDA receptors and PSD-95 from rat brain. Detergent extracts of rat cerebral cortex synaptosomal membranes were immunoprecipitated with PSD-95 or SAP97 antibodies or with no primary antibodies. Immunoprecipitates were then immunoblotted for α -actinin-2, NR2B, NR1, PSD-95, SAP97 and CaM kinase II α -subunit. First lane (extract) contains 5% of input used for the immunoprecipitation.

α -actinins belong to the spectrin/dystrophin family of proteins^{14,15}, which is characterized by an N-terminal actin-binding domain and a central rod domain consisting of multiple spectrin-like repeats.

Clone A2.10 lacks the actin-binding domain of α -actinin-2, but extends from a region within the first spectrin-like repeat to the 3' untranslated region (UTR) of α -actinin-2 (Fig. 1a). Deletion of the C-terminal region of α -actinin-2, including the EF hands, did not affect its interaction with NR1 C-terminal tail (C0-C1-C2 or C0-C2), indicating that the central rod domain of α -actinin-2 is sufficient for binding to NR1 (Fig. 1a). A deletion extending into repeat 4 of the rod domain abolished the interaction, but whether this deletion disrupted the NR1 binding site or affected the anti-parallel dimerization of the rod domain remains to be determined^{14,15}. NR1 showed no detectable binding to α -actinin-3.

The C0 segment of the NR1 C-terminal tail was both necessary and sufficient for interaction with α -actinin-2 (Fig. 1b), suggesting that the C0 segment contains the α -actinin-2 binding site. The membrane-proximal C0 region is present in all known C-terminal splice variants of NR1 (refs 12, 16), so the interaction of α -actinin-2 with NR1 presumably applies to all splice forms of NR1. Two-hybrid assays revealed that α -actinin-2 also bound to the C-terminal 122 residues, but not to the last 30 residues, of the cytoplasmic tail of the NMDA receptor subunit NR2B (Fig. 1a). In contrast, there was no detectable binding of α -actinin-2 to the cytoplasmic tail of NR2A (Fig. 1a). As expected, however, all NR2B and NR2A tail constructs interacted with PSD-95, which recognizes the very C-terminal ESDV sequence motif of NR2 subunits^{9,10}. Thus, α -actinin binds to NR2B at a site distinct from that recognized by the PDZ domains of PSD-95.

A direct association between α -actinin-2 and NMDA receptor subunits was confirmed by *in vitro* binding of recombinant fusion proteins in a filter overlay assay (Fig. 2a). α -Actinin-2 binds to glutathione-S-transferase (GST) fusion proteins of the cytoplasmic tails of NR1 (C0-C1-C2 and C0-C2) and of NR2B, but not to the cytoplasmic tail of Kv1.4 or NR2A. By contrast, PSD-95, binds to Kv1.4 and NR2 subunits but not to NR1 (Fig. 2a).

A rabbit polyclonal anti-peptide antibody specific to α -actinin-2 (termed 4B2) recognized a band of M_r ~100K on immunoblots of rat brain membranes, consistent with the predicted size of α -actinin-2 (ref. 13) (Fig. 2b, c). When coupled to beads, GST fusion proteins of the cytoplasmic tails of NR1A and NR1C were able specifically to affinity-purify ('pull-down') α -actinin-2 protein from brain extracts, but not chapsyn-110 (ref. 17) or other members of the PSD-95 family of proteins (Fig. 2b). Conversely, the Kv1.4 C-terminal tail selectively 'pulled-down' members of the PSD-95 family, including chapsyn-110 (Fig. 2b) and PSD-95 (not shown), but not α -actinin-2.

Upon subcellular fractionation, α -actinin-2 and NMDA receptor subunits copurify into the postsynaptic density fraction where they are both resistant to extraction with Triton X-100 and Sarkosyl detergents (Fig. 2c). Thus, like NMDA receptors and PSD-95, α -actinin-2 appears to be tightly associated with the postsynaptic density. Moreover, α -actinin-2 could be coimmunoprecipitated with NR1 and NR2B proteins by PSD-95-specific antibodies (Fig. 2d), consistent with a direct or indirect association of these proteins in brain membranes. The relative specificity of this coimmunoprecipitation is shown by first, the lack of NMDA receptor/ α -actinin-2 precipitation with antibodies against SAP97, a presynaptic relative of PSD-95 (refs 17, 18); and second, by the absence of coimmunoprecipitated calmodulin (CaM) kinase II α -subunit, an abundant component of the postsynaptic density¹⁹ (Fig. 2d).

α -Actinin-2 was localized by fluorescence immunohistochemistry in rat brain sections using rabbit 4B2 antibodies and a mouse monoclonal antibody (EA-53) specific for α -actinin-2 and α -actinin-3. α -actinin-3 is not expressed in the rat brain (M.W. and A.H.B., unpublished observations), therefore EA-53 should recognize only α -actinin-2 in central neurons. Immunostaining with both these independent antibodies revealed that α -actinin-2 was highly concentrated in dendritic spines in rat brain neurons (Fig. 3a-c).

In cultured hippocampal neurons, α -actinin-2 colocalized closely with NR1 (Fig. 3d-f) and with PSD-95 in dendritic spines (Fig. 3g-i), at presumed synaptic sites (that is, these puncta were apposed to the presynaptic markers synaptophysin and SV2 (not shown)). In addition, F-actin was itself highly concentrated in spines and colocalized with α -actinin-2 (Fig. 3j-l). The colocalization of α -actinin-2, NMDA receptors and PSD-95 in dendritic spines is consistent with the association of these proteins in a coimmunoprecipitable complex at postsynaptic sites (Fig. 2d).

As α -actinin is a known actin-binding protein, a direct interaction between α -actinin-2 and NR1/NR2B would link NMDA

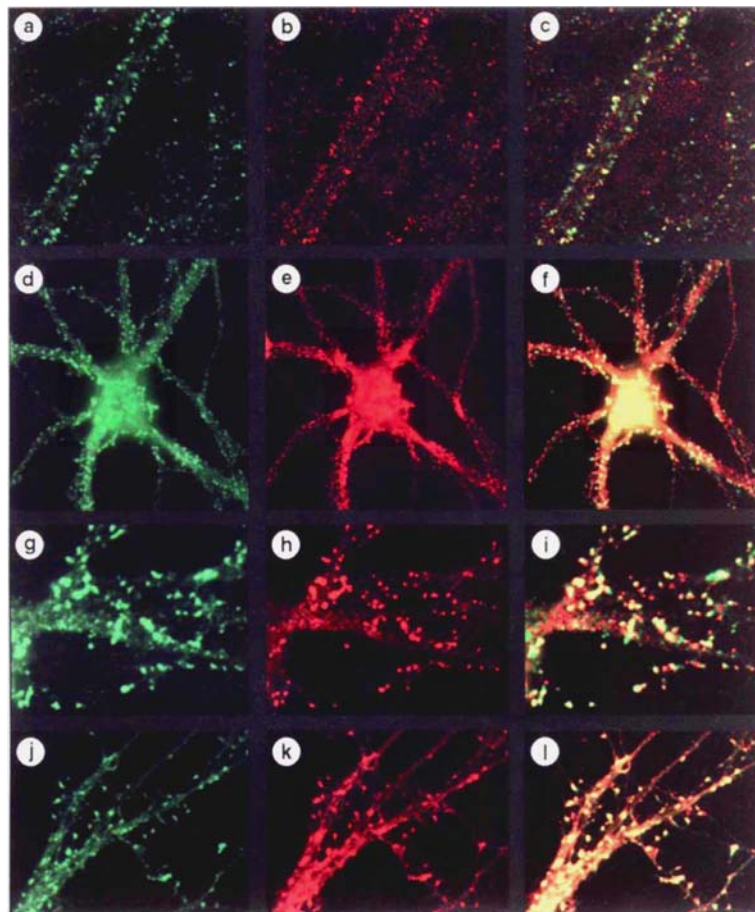


Figure 3 Colocalization of α -actinin-2 with NR1, PSD-95 and F-actin in dendritic spines. **a–c**, α -Actinin-2 in apical dendrites of cortical layer-5 pyramidal neurons of rat brain visualized using two independent α -actinin-2 antibodies: mouse monoclonal EA-53 (green; **a**), and rabbit polyclonal 4B2 (red; **b**). **d–l**, Colocalization of α -actinin-2 with NR1, PSD-95 and F-actin in cultured hippocampal neurons.

d–f, Colocalization of α -actinin-2 (green; **d**) and NR1 (red; **e**). **g–i**, Colocalization of α -actinin-2 (green; **g**) and PSD-95 (red; **h**). **j–l**, Colocalization of α -actinin-2 (green; **j**) and F-actin (stained with rhodamine-conjugated phalloidin; **k**). Composite images show colocalization of signals (yellow; **c, f, i** and **l**).

receptors to postsynaptic actin and could provide a molecular basis for the dependence of NMDA receptor function on the integrity of the actin cytoskeleton². Ca^{2+} /CaM can bind to the C0 and C1 segments of the NR1 cytoplasmic tail and inhibit the activity of recombinant NR1/NR2A NMDA receptors²⁰. α -actinin-2 binds to NR1 and NR2B with a K_D of 30–100 nM (M.W. and J.L., unpublished observations), compared with a K_D of ~90 nM reported for CaM binding to the C0 segment of NR1 (ref. 20). α -Actinin-2 binding to NR1 is directly antagonized by calmodulin in a Ca^{2+} -dependent fashion: half-maximal inhibition of α -actinin-2 binding was seen at ~1–3 μM Ca^{2+} /CaM for either the C0-C1-C2 or the C0-C2NR1 cytoplasmic tail variant (Fig. 4, and data not shown). α -Actinin-2 binding to NR1 was antagonized by calmodulin at 100 μM but not at 100 nM free Ca^{2+} (data not shown), suggesting that α -actinin-2 may bind to NR1 at resting intracellular Ca^{2+} levels *in vivo*. As calmodulin is abundant in brain and can be coimmunoprecipitated with NMDA receptors²⁰, our results indicate that Ca^{2+} /CaM may displace α -actinin-2 from NR1 subunits in response to postsynaptic Ca^{2+} influx, perhaps through activated NMDA receptors themselves. Such a mechanism could contribute not only to Ca^{2+} -dependent inactivation and rundown of NMDA receptors², but may also lead to Ca^{2+} -dependent detachment of NMDA receptors from the actin cytoskeleton and their redistribution during synaptic activity.

Ca^{2+} /calmodulin does not bind to the NR2B cytoplasmic tail and hence has no direct effect on NR2B interaction with α -actinin-2

(Fig. 4). Thus, NMDA receptors with distinct NR2 subunit compositions may be differentially regulated by Ca^{2+} /calmodulin and the cytoskeleton. □

Methods

Yeast two-hybrid screening. Yeast two-hybrid screening and assays were done as described²¹. About 2×10^6 clones were screened using a human brain cDNA library (Clontech) constructed in the Gal4-activation domain vector pGAD10. The C-terminal tail constructs of NR1 splice variants, NR2B, NR2A and Kv1.4, were generated by PCR with specific primers and subcloned into pBHA to obtain LexA fusions¹⁰. Deletion constructs of α -actinin-2 were made by PCR using specific primers and subcloned into pGAD10 to generate Gal4-activation-domain fusions.

Antibodies. α -Actinin-2-specific rabbit polyclonal antibodies were raised against a unique peptide sequence within the N terminus (NQIEPGVQYNY-VYDEDE) of α -actinin-2 and affinity-purified. These antibodies do not crossreact with any of the other known α -actinin isoforms (M.W. and A.H.B., unpublished results). Antibodies against chapsyn-110 (ref. 17), SAP97 (ref. 17), PSD-95 (ref. 21) and NR2B⁸ have been described. Anti-T7.Tag antibodies (Novagen), monoclonal anti-calmodulin antibody (UBI), monoclonal anti- Ca^{2+} /calmodulin-dependent protein kinase II antibody (Chemicon), and anti-NR1 monoclonal antibodies 54.1 (PharMingen) were used for immunoblotting at 1 $\mu\text{g ml}^{-1}$.

Filter overlay, 'pull-down' assays, immunoblotting and immunoprecipitation. Filter overlay binding was assayed as described^{10,21,22}. For affinity-purification ('pull-down') of α -actinin-2 from rat brain, 1% Triton

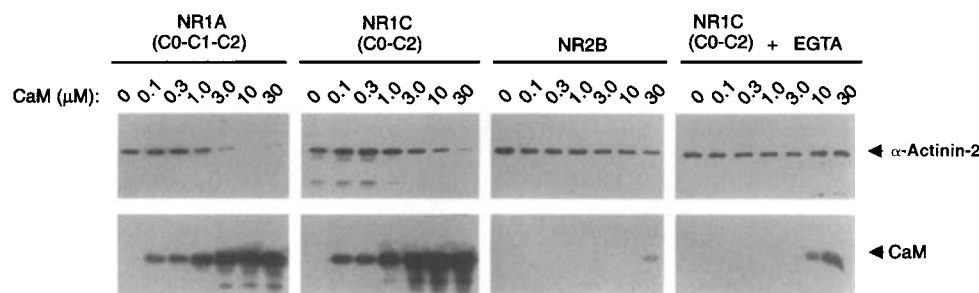


Figure 4 Competition between α -actinin-2 and Ca^{2+} /calmodulin for binding to the C-terminal tails of NR1A and NR1C. GST-fusion proteins (2 μg) of the C-terminal tails of NR1A, NR1C or NR2B bound to glutathione-Sepharose beads were incubated with a fixed concentration of hexahistidine-tagged fusion protein of α -actinin-2 (150 nM) and increasing concentrations of calmodulin (CaM). Bound

proteins were eluted and immunoblotted for α -actinin-2 using anti-T7Tag antibodies, and for calmodulin with a monoclonal anti-calmodulin antibody. Binding reactions containing 5 mM EGTA (+EGTA) are indicated. All other binding reactions contained 2 mM CaCl_2 .

X-100 extracts of rat brain (200 μg protein) were incubated with glutathione-Sepharose beads (Pharmacia) bound to GST-fusion proteins (100 μg) at 4 $^{\circ}\text{C}$ for 1 h. After washing with phosphate-buffered saline containing 0.1% Triton X-100, bound proteins were eluted with SDS-sample buffer and detected by immunoblotting. Coimmunoprecipitation of α -actinin-2, NR2B, NR1 and PSD-95 complexes from rat brain was done on SDS-extracted rat brain membranes as described²³. For α -actinin-2/calmodulin competition, purified hexahistidine-tagged α -actinin-2 and glutathione-Sepharose bound GST-fusion proteins were incubated with purified calmodulin (Calbiochem) in 25 mM HEPES, 120 mM KCl, 1 mM EDTA, 0.2% Triton X-100, pH 7.6, and either 2 mM CaCl_2 or 5 mM EGTA at 4 $^{\circ}\text{C}$ for 1 h. All proteins were visualized using peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL, Amersham).

Immunocytochemistry. Brain immunohistochemistry was done as described¹⁷. Hippocampal neuronal cultures were prepared as described²⁴. Neurons were fixed and permeabilized 18–35 d after plating, either with cold methanol or with 4% paraformaldehyde and 0.25% Triton X-100. Rhodamine-conjugated phalloidin was used at a concentration of 100 ng ml^{-1} . Primary antibodies were used at the indicated concentrations: 4B2 (1 μg ml^{-1}), EA-53 (1:20,000; Sigma), anti-NR1 monoclonal antibodies 54.1 (2 μg ml^{-1} ; Phar-Mingen) and were visualized with FITC-, Cy3-, and biotin-conjugated secondary antibodies (2.5 μg ml^{-1} ; Jackson ImmunoResearch) and Texas red-conjugated streptavidin (500 ng ml^{-1} ; Vector Labs). Fluorescent images of cells were captured on a Photometrics cooled CCD camera mounted on a Zeiss Axioskop microscope (cultured hippocampal neurons), or on a Biorad Confocal MRC 1000 (rat brain sections).

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Crosstalk between G proteins and protein kinase C mediated by the calcium channel α_1 subunit

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The modulation of voltage-dependent Ca^{2+} channels at presynaptic nerve terminals is an important factor in the control of neurotransmitter release and synaptic efficacy. Some terminals contain multiple Ca^{2+} -channel subtypes (N and P/Q)^{1–3}, which are differentially regulated by G-protein activation^{4–8} and by protein kinase C (PKC)-dependent phosphorylation^{9–11}. Regulation of channel activity by crosstalk between second messenger pathways has been reported^{12,13}, although the molecular mechanisms underlying crosstalk have not been described. Here we show that crosstalk occurs at the level of the presynaptic Ca^{2+} -channel complex. The α_1 subunit domain I–II linker, which connects the first and second transmembrane domains, contributes to the PKC-dependent upregulation of channel activity, while G-protein-dependent inhibition occurs through binding of $\text{G}\beta\gamma$ to two sites in the I–II linker. Crosstalk results from the PKC-dependent phosphorylation of one of the $\text{G}\beta\gamma$ binding sites which antagonizes $\text{G}\beta\gamma$ -induced inhibition. The results provide a mechanism for the highly regulated and dynamic control of neurotransmitter release that depends on the integration of multiple presynaptic signals.

Coexpression of a Ca^{2+} -channel β subunit antagonizes the G-protein-dependent inhibition of neuronal Ca^{2+} currents^{8,14}. Because the Ca^{2+} -channel β subunit binds to the α_1 subunit domain I–II cytoplasmic linker¹⁵, we tested whether this region also contributes to G-protein modulation. Expression of α_{1A} and α_{1B} subunits (with α_2 and β_{1b}) in HEK cells results in P/Q-type and N-type currents

that are inhibited by stimulation of an endogenous somatostatin receptor (Fig. 1). We found that α_{1B} currents are blocked by somatostatin significantly more than α_{1A} currents ($65 \pm 4.0\%$, $n = 10$, versus $22.6 \pm 2.8\%$, $n = 15$). To determine whether the domain I-II linker contributes to the larger degree of inhibition observed for α_{1B} currents, we examined a chimaeric α_1 subunit in which the α_{1A} domain I-II linker was replaced by the α_{1B} I-II linker (α_{1AB} ; Fig. 1b). Transient expression of the α_{1AB} chimaera results in P/Q-type currents in which the degree of inhibition by somatostatin was increased by nearly 100% compared with that for α_{1A} currents (to $41.0 \pm 3.3\%$, $n = 9$; Fig. 1a, c). Expression in *Xenopus* oocytes with the μ -opioid receptor resulted in a similar qualitative and quantitative inhibition upon application of the opioid agonist DAMGO, so the observed effects were not due to the particular expression system or receptor type (Fig. 1c; α_{1A} , $19.4 \pm 1.0\%$, $n = 54$; α_{1B} , $55.4 \pm 1.4\%$, $n = 43$; α_{1AB} , $38.1 \pm 2\%$, $n = 27$).

The inhibition of Ca^{2+} currents by activation of G-protein-coupled receptors can be relieved by application of a strong depolarizing prepulse resulting in an apparent facilitation of whole-cell currents⁴⁻⁸. HEK cells stably expressing α_{1B} , α_2 and β_{1B} N-type Ca^{2+} channels (α_{1B} HEK cells) do not exhibit facilitation upon application of a positive depolarizing prepulse to +150 mV for 50 ms before a test pulse to +20 mV, indicating that α_{1B} channels are not tonically inhibited (current ratio, with/without prepulse, 0.95 ± 0.04 , $n = 25$) (Fig. 2a). In contrast, inclusion of purified G $\beta\gamma$ subunits (10 nM) in the patch pipette results in N-type currents which are facilitated by ~30% upon application of a depolarizing prepulse before a test pulse to +20 mV (current ratio, with/without prepulse, 1.30 ± 0.04 , $n = 19$). Consistent with the modulation of N-type currents in native and recombinant cells¹⁶⁻¹⁸, this prepulse relief of G $\beta\gamma$ inhibition is voltage dependent, and is significantly larger at more hyperpolarized potentials (Fig. 2b). Both the magnitude and voltage dependence of the G $\beta\gamma$ -induced prepulse facilitation parallel the prepulse facilitation of α_{1B} currents inhibited by the application of 100 nM somatostatin in the absence of exogenous G $\beta\gamma$ subunits (data not shown). Inclusion in the pipette of 10 nM G $\beta\gamma$ heated to 95 °C for 10 min results in a significant decrease in the ability of G $\beta\gamma$ to render α_{1B} currents capable of facilitation (current ratio, with/without prepulse, 1.09 ± 0.04 , $n = 6$). Similarly, inclusion of a mixture of purified G α_o and G α_i subunits in the pipette does not result in N-type

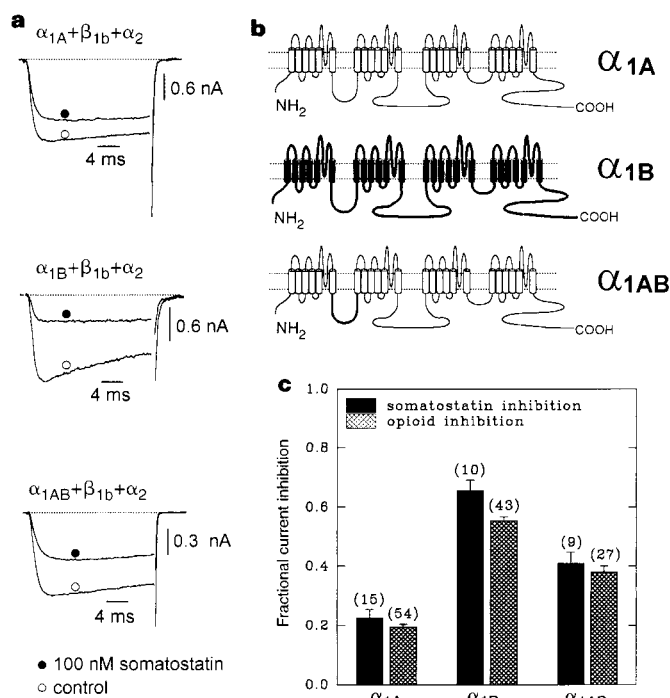


Figure 1 The Ca^{2+} -channel α_1 subunit domain I-II linker contributes to G-protein-dependent inhibition. **a**, Whole-cell current traces recorded from $\alpha_{1A} + \alpha_2 + \beta_{1B}$, $\alpha_{1B} + \alpha_2 + \beta_{1B}$ and $\alpha_{1AB} + \alpha_2 + \beta_{1B}$ channels transiently expressed in HEK cells in the presence (filled symbol) and absence (open symbol) of 100 nM somatostatin. Currents were evoked by stepping from -100 mV to the peak of the $I-V$ relation. The domain I-II linker partly confers the higher G-protein sensitivity of α_{1B} onto α_{1A} . **b**, Schematic representation of the α_{1A} and α_{1B} subunits and the chimaeric subunit in which the domain I-II linker from α_{1B} was substituted into α_{1A} (α_{1AB}). **c**, Summary of the G-protein inhibition of α_{1B} , α_{1A} and α_{1AB} ($+\alpha_2 + \beta_{1B}$), expressed either in HEK cells using the somatostatin pathway (black bars) or in *Xenopus* oocytes with a coexpressed μ -opioid receptor activated by 1 μM DAMGO (hatched bars).

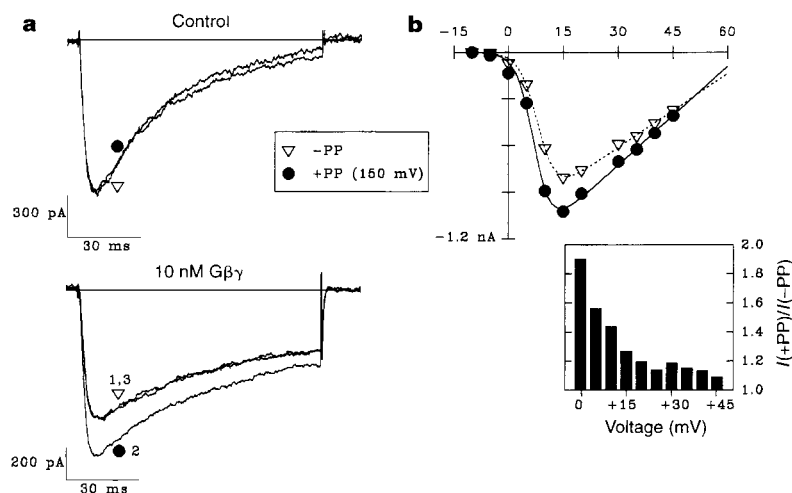


Figure 2 Purified G $\beta\gamma$ in the patch pipette renders α_{1B} calcium currents susceptible to prepulse facilitation. **a**, Whole-cell current records obtained from $\alpha_{1B} + \alpha_2 + \beta_{1B}$ stably expressed in HEK cells with (filled circles) and without (open triangles) a 50 ms prepulse (PP) to +150 mV before a test pulse to +20 mV. Top, no prepulse effect is observed in control cells. Bottom, with 10 nM G $\beta\gamma$ in the patch pipette, a prepulse to +150 mV produces an ~30% increase in

the peak current amplitude. The numbers indicate the order in which the traces were recorded (1, -PP; 2, +PP; 3, -PP), and show that run-up did not occur. **b**, Current-voltage relation of the G $\beta\gamma$ -induced facilitated current. The prepulse effect occurs over the complete range of test potentials and is more pronounced at hyperpolarizing potentials (inset, current ratio, $I(+PP)/I(-PP)$).

currents capable of prepulse facilitation (current ratio, with/without prepulse, 0.97 ± 0.03 , $n = 13$). Taken together, these data indicate that, in α_{1B} HEK cells, purified G $\beta\gamma$ subunits mimic the G-protein-dependent inhibition of whole-cell N-type currents observed upon activation of G-protein coupled receptors.

It has generally been assumed that the G-protein modulation of Ca $^{2+}$ channels through membrane-delimited pathways involves a direct interaction of G proteins with the Ca $^{2+}$ -channel complex, but no direct evidence for this has been presented. To determine whether G $\beta\gamma$ binds directly to the Ca $^{2+}$ -channel α_1 subunit, we constructed a series of fusion proteins to various regions of α_{1B} . Protein overlay assays were performed with *in vitro* translated ^{35}S -radiolabelled G $\beta_1\gamma_2$, G α_o (activated with 100 μM GTP- γS) and the Ca $^{2+}$ -channel β_{1b} subunit. Overlay with G $\beta\gamma$ results in binding to the α_{1B} domain I–II linker, and not to the domain II–III linker, domain III S5–S6, a region carboxyl to domain IV S6 or the maltose binding protein–Lac Z fusion control (Fig. 3a). Consistent with results showing that G α subunits do not directly modulate N- and P/Q-type currents^{16,17}, G α_o does not bind directly to the domain I–II linker or to the other regions tested.

The α_1 subunit domain I–II linker possesses a consensus motif for binding of G $\beta\gamma$ (QXXER)¹⁹ that is contained within the site responsible for direct binding of the Ca $^{2+}$ -channel β subunit (Fig. 3b). To define the regions of the I–II linker crucial for G $\beta\gamma$ interaction, we examined a series of synthetic peptides corresponding to various regions of the α_{1A} and α_{1B} domain I–II linkers (Fig. 3b). With 2 μM peptide in the pipette, the α_{1B} peptides α_{1B} (445–463) and α_{1B} (390–409) have no significant effect on the prepulse facilitation induced by G $\beta\gamma$ (α_{1B} (445–463), with/without prepulse, 1.29 ± 0.05 , $n = 15$; α_{1B} (390–409), with/without prepulse, 1.24 ± 0.05 , $n = 12$) (Fig. 3c). In contrast, prepulse facilitation is effectively blocked in the presence of peptides α_{1B} (353–371) (with/without prepulse, 1.05 ± 0.02 , $n = 10$), α_{1B} (372–389) (with/without prepulse, 0.99 ± 0.02 , $n = 10$) and α_{1B} (410–428) (with/without prepulse, 1.03 ± 0.03 , $n = 9$). These results indicate that certain peptides are able to quench the exogenously added G $\beta\gamma$, presumably through direct competition for binding. The α_{1B} (390–409) peptide has no effect on G $\beta\gamma$ prepulse facilitation, whereas flanking peptides on the amino-terminal side (α_{1B} (353–371)) and α_{1B} (372–389) and carboxy-terminal side (α_{1B} (410–428)) block G $\beta\gamma$ -induced prepulse facilitation. This suggests that G $\beta\gamma$ binding involves two separable binding sites in the domain I–II linker, and is consistent with ref. 20.

Because α_{1A} P/Q-type currents are also inhibited by G $\beta\gamma$, we tested whether peptides against the α_{1A} domain linker could also block the G $\beta\gamma$ modulation of N-type currents in α_{1B} HEK cells. The α_{1A} peptide α_{1A} (384–403), which partly overlaps α_{1B} (372–389) and α_{1B} (390–409) and contains the consensus G $\beta\gamma$ binding site QXXER, effectively inhibits G $\beta\gamma$ -dependent prepulse facilitation (1.02 ± 0.04 , $n = 13$) (Fig. 3c). The peptide α_{1A} (416–434), which shares ~47% identity with α_{1B} (410–428), also inhibits G $\beta\gamma$ -induced prepulse facilitation (1.00 ± 0.03 , $n = 17$). The α_{1A} (451–469) peptide, which shares ~68% identity with α_{1B} (445–463), also did not significantly affect G $\beta\gamma$ -dependent prepulse facilitation (1.28 ± 0.06 , $n = 9$). Taken together, the data suggest that, although domain I–II linkers of the α_{1A} and α_{1B} channels are not identical in primary sequence, the mechanism of G $\beta\gamma$ interaction is likely to be similar in both channel types.

PKC-dependent phosphorylation has been shown to modulate Ca $^{2+}$ -channel activity in many types of neurons^{9,10,21}. Crosstalk (or convergence) occurs between the PKC-dependent upregulation and G-protein-mediated inhibition of Ca $^{2+}$ current^{12,13}. Crosstalk is exhibited by N-type currents expressed in stable α_{1B} HEK cells (Fig. 4a). Application of 1 μM somatostatin results in an ~50% block ($54 \pm 4.9\%$, $n = 9$; Fig. 4a). Stimulation of PKC with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) upregulates α_{1B} currents, as previously described¹¹, but subsequent application of

somatostatin produces only ~20% inhibition ($22.0 \pm 3.0\%$, $n = 6$; Fig. 4a), indicating that N-type currents are modulated by crosstalk between the two second messenger pathways that is similar to that in native cells^{12,13}. Pretreatment with the PKC inhibitor staurosporine abolishes the antagonistic effect of TPA on somatostatin action, and minimizes the possibility of any nonspecific phorbol ester effects (data not shown).

Substitution of the α_{1B} domain I–II linker into α_{1A} confers PKC-dependent upregulation on α_{1A} currents¹¹, and this region is implicated in G $\beta\gamma$ modulation (Figs 1 and 3). We therefore hypothesized that direct phosphorylation of domain I–II linker may contribute to the PKC-dependent attenuation of the G-protein-induced inhibition (Fig. 4a). The α_{1B} domain I–II linker fusion protein is a substrate for PKC-dependent phosphorylation *in vitro* (Fig. 4b). Furthermore, the α_{1B} (410–428) and α_{1B} (445–463) peptides, and the α_{1A} (416–434) and α_{1A} (451–469) peptides, all of which contain consensus PKC sites, are substrates for PKC-dependent phosphorylation. Peptides that do not contain consensus PKC sites are not substrates for PKC phosphorylation (compare Figs 3b and 4b).

To determine whether phosphorylation of the domain I–II

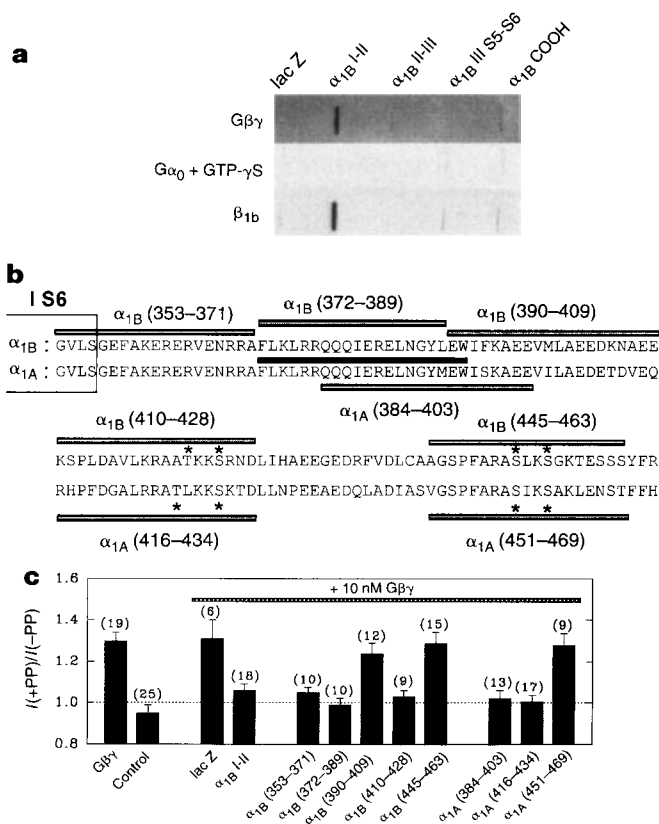


Figure 3 G $\beta\gamma$ binds to the α_1 subunit domain I–II linker. **a**, Autoradiogram of overlay binding of *in vitro* ^{35}S -radiolabelled G $\beta\gamma$, G α_o (+100 μM GTP- γS) and Ca $^{2+}$ -channel β_{1b} to fusion proteins directed against the Lac-Z fusion control and the α_{1B} subunit. The α_{1B} fusions are: domain I–II linker, residues 360–481; domain II–III linker, 875–1047; domain III S5–S6, 1304–1398; carboxyl region, 1730–1858. **b**, Primary sequence of α_{1B} and α_{1A} domain I–II linkers and synthetic peptides (open bars). The filled bar denotes the Ca $^{2+}$ -channel β -subunit binding site, and asterisks indicate PKC consensus sites. **c**, Ratio of peak whole-cell currents recorded from HEK cells stably expressing α_{1B} + α_2 + β_{1b} with a prepulse +150 mV (+PP) to those in the absence of prepulse (–PP). The first two panels on the left represent the ratio +PP/–PP for inclusion of 10 nM G $\beta\gamma$ in the patch pipette compared with that for no G $\beta\gamma$ in the pipette. The remaining panels show the effects of inclusion of the fusion proteins and synthetic peptides (2 μM) in the presence of 10 nM G $\beta\gamma$. The holding potential was –100 mV, and test potential +20 mV.

residues could account for the observed crosstalk, we phosphorylated the I–II linker fusion protein and the synthetic peptides *in vitro*, and compared their effects on G $\beta\gamma$ -dependent prepulse facilitation. Phosphorylation of the I–II linker fusion protein and the α_{1B} (410–428) or α_{1A} (416–434) peptides relieves the block of G $\beta\gamma$ -induced prepulse facilitation compared with the unphosphorylated substrates (Fig. 4c, d). In contrast, the α_{1B} (445–463) and α_{1A} (451–469) peptides do not affect G $\beta\gamma$ -induced prepulse facilitation, regardless of their phosphorylation state. These results suggest that attenuation of the G $\beta\gamma$ response is mediated by the PKC-dependent phosphorylation of residues covered by α_{1B} (410–428) and α_{1A} (416–434).

It is now well established that the GIRK family of inwardly rectifying K $^{+}$ channels are activated by direct binding of G $\beta\gamma$ ^{22–25}. Our data demonstrate that the membrane-delimited inhibition of Ca $^{2+}$ channels occurs through a direct binding of G $\beta\gamma$ to the Ca $^{2+}$ -channel α_1 subunit domain I–II linker (see ref. 20). Both G $\beta\gamma$ binding and Ca $^{2+}$ -channel β -subunit binding occur in an over-

lapping region of the I–II linker and the results suggest a possible explanation for the observation that Ca $^{2+}$ -channel β -subunit coexpression attenuates direct G-protein inhibition^{8,14}. Because substitution of the α_{1B} domain I–II linker only partially confers α_{1A} currents with the higher degree of inhibition associated with α_{1B} , we cannot exclude the possibility that additional regions may also contribute to G-protein inhibition. Our results also demonstrate that the crosstalk or convergence of second messenger pathways acting on Ca $^{2+}$ currents occurs within the channel complex. The data suggest a model in which G $\beta\gamma$ interaction in the domain I–II linker is directly affected by the PKC-dependent phosphorylation of residues within the G $\beta\gamma$ binding site. Both the α_{1A} (P/Q-type) and α_{1B} (N-type) Ca $^{2+}$ channels have been implicated in triggering neurotransmitter release, and both are coexpressed at a subset of terminals^{1–3,26,27}. Crosstalk would allow the temporal integration of second messenger signals and may be particularly important at terminals that receive multiple synaptic inputs. □

Methods

Electrophysiological recording. Three expression systems were used to examine α_{1A} P/Q-type and α_{1B} N-type currents: (1) whole-cell patch-clamp recordings on stably transfected HEK cells expressing rat brain α_{1B} , α_2 and β_{1B} subunits^{8,11}; (2) whole-cell patch-clamp recordings on HEK cells transiently expressing α_{1A} , α_2 and β_{1B} subunits^{8,11}, α_{1B} , α_2 and β_{1B} subunits and an α_{1B} domain I–II linker chimera¹¹, α_2 and β_{1B} subunits; and (3) two microelectrode recordings on *Xenopus* oocytes transiently expressing the various Ca $^{2+}$ -channel subunit combinations. For patch recordings the internal pipette solution contained (in mM) 105 CsCl, 25 TEA-Cl, 1 CaCl $_2$, 11 EGTA, 10 HEPES, pH 7.2. The external recording solution was (in mM) 20 BaCl $_2$, 1 MgCl $_2$, 10 HEPES, pH 7.2, 40 TEA-Cl, 10 glucose, 65 CsCl. Unless otherwise stated, currents were elicited from a holding potential of -100 mV to a test potential of $+20$ mV. G-protein-induced facilitation was assessed by a 50-ms prepulse to $+150$ mV before the test pulse. For transient expression in HEK cells, cDNAs for α_{1A} or α_{1B} or α_{1A} plus α_2 and β_{1B} subunits and the jellyfish green fluorescent protein at a molar ratio of 1:1:1:0.1 were co-transfected into HEK cells by a standard calcium-phosphate protocol. The effect of somatostatin was assessed using a 0.2-Hz train of step depolarizations from a holding potential of -100 mV to the peak of the current–voltage relation. Purified bovine G α (mixture of G α_i and G α_o) and G $\beta\gamma$ were stored frozen in 10 mM HEPES, pH 8.0, 200 mM NaCl, 0.5% Na cholate, 1 mM DTT, diluted to 1 μ M in 0.1% CHAPS, and then to a final concentration of 10 nM into the internal recording solution immediately before recording. For oocyte recording, *Xenopus* oocytes were prepared and nuclear injections with cDNAs performed^{8,11}. Macroscopic currents were recorded in a solution containing (in mM) 40 Ba(OH) $_2$, 25 TEA-OH, 25 NaOH, 2 CsCl, 5 HEPES titrated to pH 7.4 with methanesulphonic acid. Modulation by the μ -opioid receptor was as described⁸. Currents were elicited from a holding potential of -100 mV to a test potential of $+20$ mV. Error bars indicate s.e.m., and significance was assessed using a Student's *t*-test.

Synthetic peptides and fusion proteins. Synthetic peptides were diluted from stocks (10 mM in water) directly in the internal pipette solution (final concentration of 2 μ M). For phosphorylation experiments, synthetic peptides (10 nmol) were phosphorylated *in vitro* for 10 min at 30 °C in a solution containing (in mM) 20 HEPES, pH 7.4, 0.34 EDTA, 0.34 EGTA, 1.67 CaCl $_2$, 1 DTT, 10 MgCl $_2$, 200 μ g μ l $^{-1}$ phosphatidyl serine, and either 5 mM cold ATP or 0.15 mM 32 P- γ -ATP (6,000 Ci mmol $^{-1}$). 32 P-radiolabelled peptides were separated on a 30% acrylamide Laemmli gel containing 0.1% SDS, dried and exposed to X-ray film for 5 min. Fusion proteins were constructed into the pMAL-C2 fusion protein vector by PCR amplification of the rat brain α_{1B} subunit cDNA: domain I–II linker amino acid, residues 360–481; domain II–III linker, residues 875–1047; domain III S5–S6, residues 1304–1398; carboxyl region past domain IV S6, residues 1730–1858. Fusion proteins were generated by transformation into *Escherichia coli* strain TB-1 followed by induction with 1 mM IPTG and affinity purification by amylose resin chromatography. *In vitro* phosphorylation of fusion proteins by PKC was as described above, using 20 nmol of each fusion protein. 32 P-radiolabelled fusion proteins were separated on an 8% acrylamide Laemmli gel containing 0.1% SDS, dried and exposed to X-ray film for 5 min.

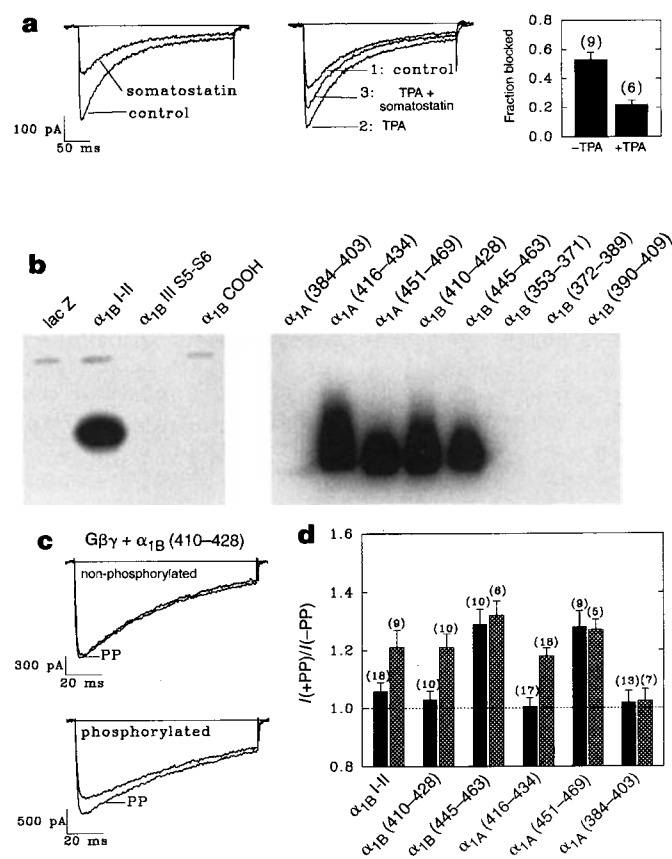


Figure 4 Crosstalk between G-protein inhibition and PKC upregulation. **a**, Inhibition of whole-cell currents in α_{1B} HEK cells by application of 1 μ M somatostatin (left). Application of 400 nM TPA upregulates α_{1B} currents (traces 1 and 2), and subsequent coapplication of 1 μ M somatostatin plus TPA decreases the TPA-induced upregulation (trace 3, middle). The holding potential was -100 mV, and the test potential $+20$ mV. TPA treatment significantly reduces the degree of somatostatin-induced inhibition (right). **b**, Autoradiograph of *in vitro* phosphorylation of α_{1B} fusion proteins and synthetic α_{1A} and α_{1B} peptides by 32 P- γ -ATP and purified PKC. **c**, Current waveforms recorded from α_{1B} HEK cells at a test potential of $+20$ mV in the presence of 10 nM G $\beta\gamma$ in the patch pipette. Inclusion of 2 μ M unphosphorylated peptide α_{1B} (410–428) in the patch pipette blocks G $\beta\gamma$ -dependent prepulse facilitation (top); *in vitro* phosphorylation of α_{1B} (410–428) relieves the block (bottom). **d**, Summary of the effects of *in vitro* phosphorylation (shaded bars) compared with non-phosphorylation (black bars) of the α_{1B} I–II linker fusion protein (2 μ M) and α_{1B} and α_{1A} I–II linker peptides (2 μ M) on G $\beta\gamma$ -dependent facilitation.

Protein overlays. Fusion proteins were dissolved in a solution of (in mM) 150 NaCl, 50 NaPO₄, pH 6.9, and 10 µg of each was applied to nitrocellulose filters using a slot-blot apparatus. Blots were blocked for 3 h at 4 °C with (in mM) 5% powdered milk, 150 NaCl, 50 NaPO₄, pH 6.9. cDNAs encoding the G-protein subunits Gα₀, Gβ₁, Gγ₂ and the Ca²⁺-channel β_{1b} were translated *in vitro* using a coupled transcription-translation kit (Promega) including 40 µCi of ³⁵S-methionine (1,000 Ci mmol⁻¹). After *in vitro* synthesis at 30 °C for 90 min, unincorporated nucleotides were removed by Sephadex G-25 spin column chromatography. The reactions were then directly added to overlay buffer (in mM, 0.5% BSA, 100 NaCl, 50 HEPES, pH 7.4, 1 DTT, 0.05% CHAPS) and incubated overnight at 4 °C. Filters were washed at 4 °C in (in mM) 5% BSA, 150 NaCl, 50 NaPO₄, pH 6.9, and exposed to X-ray film. Each overlay was performed at least 4 times with similar results. Longer exposures (up to 48 h) did not exhibit any specific labelling of Gβ₁γ₂, Gα₀ or β_{1b} to any other regions of the α_{1b} subunit than that shown in Fig. 3a.

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Direct binding of G-protein βγ complex to voltage-dependent calcium channels

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Voltage-dependent Ca²⁺ channels play a central role in controlling neurotransmitter release at the synapse^{1,2}. They can be inhibited by certain G-protein-coupled receptors, acting by a pathway intrinsic to the membrane^{3–6}. Here we show that this inhibition

results from a direct interaction between the G-protein βγ complex and the pore-forming α₁ subunits of several types of these channels⁷. The interaction is mediated by the cytoplasmic linker connecting the first and second transmembrane repeats. Within this linker, binding occurs both in the α₁ interaction domain (AID)⁸, which also mediates the interaction between the α₁ and β subunits of the channel, and in a second downstream sequence. Further analysis of the binding site showed that several amino-terminal residues in the AID are critical for Gβγ binding, defining a site distinct from the carboxy-terminal residues shown to be essential for binding the β-subunit of the Ca²⁺ channel⁹. Mutation of an arginine residue within the N-terminal motif abolished βγ binding and rendered the channel refractory to G-protein modulation when expressed in *Xenopus* oocytes, showing that the interaction is indeed responsible for G-protein-dependent modulation of Ca²⁺ channel activity.

In vitro translation of G-protein subunits (³⁵S-α₀, ³⁵S-β₁ and ³⁵S-γ₂) yielded products of relative molecular mass (M_r) 40K, 32K and 8K (Fig. 1a), respectively. These G-protein probes were used to study their interaction with glutathione S-transferase (GST) fusion proteins containing cytoplasmic loops (Fig. 1b, left) of the neuronal α_{1A} voltage-dependent Ca²⁺ channel¹⁰. The ³⁵S-Gβ₁γ₂ complex interacts with a GST fusion protein expressing the cytoplasmic linker connecting repeats I and II of the α_{1A} subunit (I–II–GST, amino acid 360–486), as demonstrated by the detection of ³⁵S-Gβ₁ on the autoradiogram (Fig. 1b, right). The I–II–GST fusion protein also binds native Gβγ complexes solubilized from rat brain in solution, as detected by immunoblot using a common anti-Gβ antibody (Fig. 1c). The binding of Gβ₁γ₂ to I–II–GST is specific, as no ³⁵S-labelled G-protein probe reacts with the control GST or a GST fusion protein expressing the II–III loop of the α_{1A} subunit (II–III–GST; Fig. 1d). Similarly, no binding is detected on fusion proteins expressing the II–III loop of rabbit α_{1C} (amino acids 772–856) and rat α_{1B} (amino acids 720–1139; data not shown). Finally, binding of the GTPγS-activated ³⁵S-Gα₀ subunit could not be detected on any of these fusion proteins (Fig. 1d). These results are thus consistent with recent findings that the Gβγ complex can regulate transfected P/Q-type Ca²⁺ channels¹¹ or N-type Ca²⁺ channels in sympathetic neurons¹².

Because N- and P/Q-type Ca²⁺ channels can be modulated by activated G proteins^{13–16}, we investigated whether the ³⁵S-Gβ₁γ₂ complex could also interact with the I–II cytoplasmic linker of several other α₁ subunit classes, including the L-type α_{1S} and α_{1C} channels, and non-L-type α_{1B} and α_{1E} channels (Fig. 2a). The ³⁵S-Gβ₁γ₂ complex also interacts with GST fusion proteins expressing the full-length I–II loops of the α_{1B} and α_{1E} channels, but not with those of the α_{1S} or α_{1C} channels (Fig. 2b). These results provide a molecular mechanism for earlier reports that α_{1A}, α_{1B} (ref. 16) and α_{1E} (ref. 14) channels are modulated by G proteins.

We have previously shown that the I–II cytoplasmic linker of all six classes of α₁ subunits contain the AID site, a sequence of 18 amino acids, of which 9 residues are universally conserved (QQ-E-L-GY-WI--E) (1–18). The AID site is both necessary and sufficient for the binding of the Ca²⁺-channel β-subunit^{8,9}. The AID sequence separates two domains in the I–II linker, one upstream of conserved length (23 amino acids; domain I, D1), and a downstream sequence of variable length (between 55 and 87 amino acids; domain II, D2). To define precisely the Gβγ binding site on the I–II linker of the α_{1A} subunit, we generated several GST fusion proteins consisting of partial I–II loop sequences (Fig. 2c). The results demonstrate that ³⁵S-Gβ₁γ₂ interacts with D1–AID and AID–D2, but not D1 alone, demonstrating the importance of AID itself in Gβγ binding (Fig. 2d). Consistent with this interpretation was the finding that ³⁵S-Gβ₁γ₂ could also interact with a fusion protein (AID–GST) expressing AID and a few flanking amino acids in D1 and D2 (Fig. 3a). Surprisingly, ³⁵S-Gβ₁γ₂ also interacts with the D2–GST fusion protein independent of AID (Fig. 2d). It is

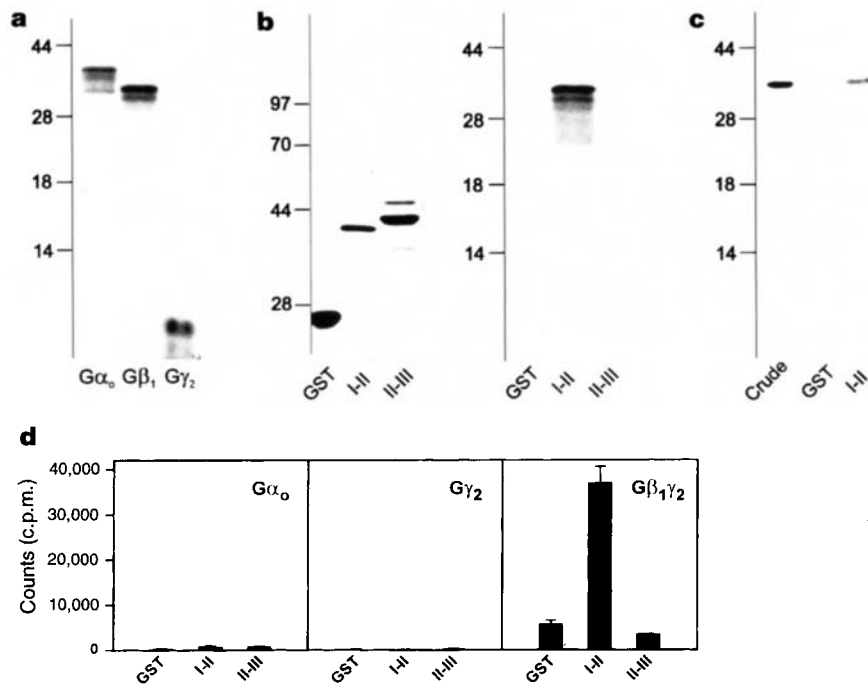


Figure 1 G-protein $\beta\gamma$ complex binds to the I-II cytoplasmic loop of the α_{1A} channel. **a**, Autoradiogram of *in vitro* translated G-protein α_o , β_1 and γ_2 subunits separated on SDS-polyacrylamide gel. **b**, Left, Coomassie blue-stained purified control GST, α_{1A} I-II and II-III loop GST fusion proteins (I-II-GST and II-III-GST) separated on a 10% SDS-polyacrylamide gel. Right, autoradiogram of bound proteins from incubation of co-translated ^{35}S - $G\beta_1\gamma_2$ with glutathione-Sepharose

beads coupled with control GST, I-II-GST and II-III-GST fusion proteins. Detection of ^{35}S - $G\gamma_2$ is hampered by its comparatively weak labelling and small size. **c**, Western blot using common G β antibody (Dupont NEN) on crude rat brain membrane (crude) or brain extract bound to GST or I-II-GST glutathione beads. **d**, Binding of ^{35}S - $G\alpha_o$, $G\gamma_2$ or co-translated ^{35}S - $G\beta_1\gamma_2$ to GST, I-II-GST and II-III-GST.

therefore concluded that the interaction of $G\beta\gamma$ with the I-II cytoplasmic linker can occur on two separate regions, the AID site (required for Ca^{2+} -channel β -subunit binding), and the D2 sequence.

We next investigated the binding properties of $G\beta\gamma$ to the AID and the D2 sequences. Analysis of the binding of AID-GST to ^{35}S - $G\beta_1\gamma_2$ demonstrates that the specific binding is saturable and occurs on a single site with an apparent K_d of 63 nM (Fig. 3a). The affinity of $G\beta\gamma$ for the AID site is thus 10- to 20-fold lower than that of the Ca^{2+} -channel β subunit¹⁷, which may predict a more labile interaction at that site. Saturation binding analysis with a D2-MBP fusion protein shows that the binding of D2 is of slightly higher affinity ($K_d = 24$ nM) than AID-GST (Fig. 3b). *In vitro* binding affinity may differ from physiological affinity owing to difference in α_1 sequence conformation and membrane localization of G proteins. Taken together, these results suggest that the D2 region is a more stable $G\beta\gamma$ attachment site than AID. To test this hypothesis, we expressed full-length I-II-GST fusion proteins containing AID mutants or AID chimaeras (D1_A-AID_{R387E}-D2_A mutant, D1_A-AID_S-D2_A and D1_A-AID_C-D2_A chimaeras) and tested their ability to bind the $G\beta\gamma$ complex. All these fusion proteins were able to interact with $G\beta\gamma$, as well as the wild-type I-II_A-GST fusion protein (Fig. 3c). In contrast, D1_A, D1_S-AID_S-D2_S (I-II loop of α_{1S}), and D1_C-AID_C-D2_C (I-II loop of α_{1C}) were unable to bind $G\beta\gamma$ (Fig. 2b, d). These results demonstrate that, although AID_A can bind $G\beta\gamma$, it is not absolutely essential for the attachment of the $G\beta\gamma$ complex to the I-II_A cytoplasmic linker.

Because both $G\beta\gamma$ and the Ca^{2+} -channel β -subunit bind to AID, it is important to define the critical AID residues involved in $G\beta\gamma$ binding. AID-GST chimaeras and mutants were purified and their binding compared with that of wild-type AID_A-GST fusion protein (all these fusion proteins lacked most of the D1 and D2 sequence). The results show that an AID_C chimaera, in which only the AID_A site is replaced by AID_C, did not bind the $G\beta\gamma$ complex (Fig. 3d), which is consistent with the results from Fig. 2b. This result suggests the

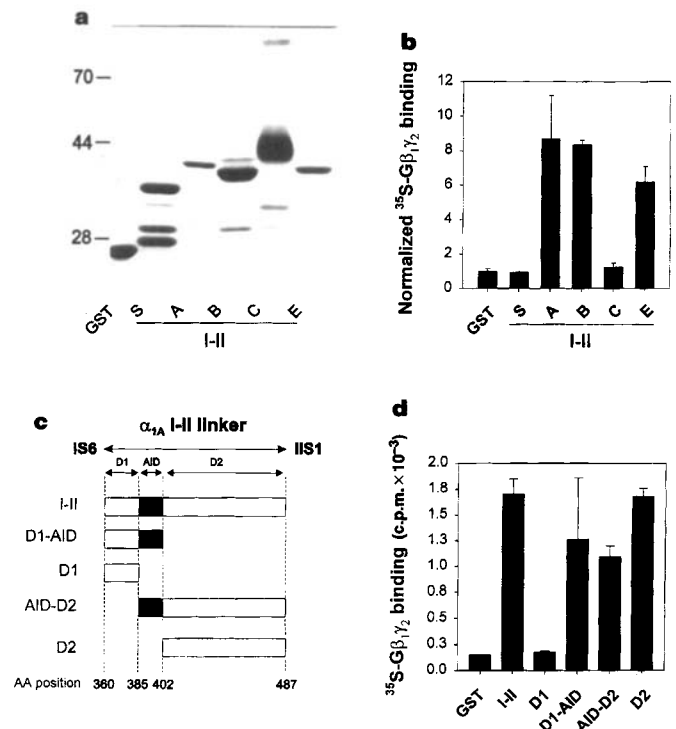


Figure 2 Interactive channel types and localization of a G-protein $\beta\gamma$ binding site on the I-II_A loop. **a**, Coomassie blue-stained 10% SDS-polyacrylamide gel of GST control and α_{1S} , α_{1A} , α_{1B} , α_{1C} and α_{1E} I-II loops expressed as GST fusion proteins (I-II_S-GST, I-II_A-GST, I-II_B-GST, I-II_C-GST and I-II_E-GST). Smaller bands are proteolytic fragments. **b**, Binding of ^{35}S - $G\beta_1\gamma_2$ to GST fusion proteins. **c**, Schematic of several constructs expressing partial sequences of the α_{1A} I-II loop. **d**, Binding of ^{35}S - $G\beta_1\gamma_2$ to GST fusion proteins expressing partial sequences of the α_{1A} I-II loop.

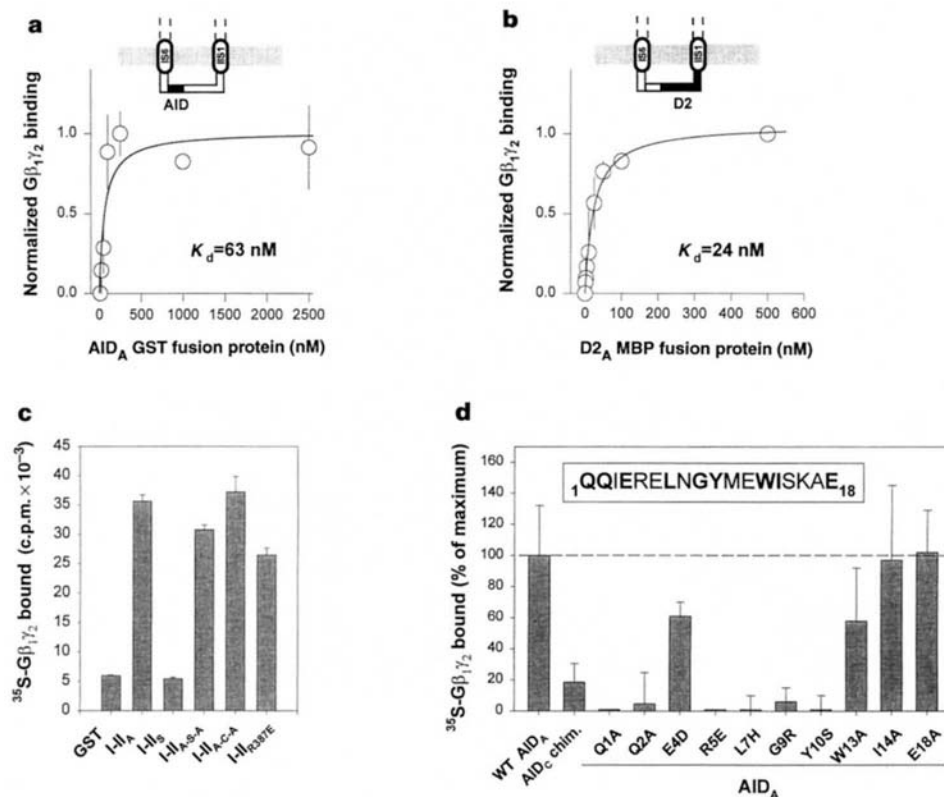


Figure 3 Binding properties of the interacting α_{1A} sequences and identification of critical AID amino acids. **a**, Saturation curve showing binding of ^{35}S -G $\beta_1\gamma_2$ to AID_A-GST. **b**, Saturation curve showing binding of D2_A-maltose binding protein (MBP) fusion protein to ^{35}S -G $\beta_1\gamma_2$. **c**, Binding of mutant or chimaeric α_{1A} I-II loop to

the G $\beta_1\gamma_2$ complex in the presence of the D2_A sequence. I-II_{A-S-A} stands for the exchange of AID_A for AID_S in the I-II_A loop. **d**, Normalized binding of ^{35}S -G $\beta_1\gamma_2$ to 250 nM of purified mutant and chimaeric AID-GST fusion proteins. Amino acids of AID_A are numbered from 1 to 18. R5E is the same mutation as R387E in **c**.

possible involvement of other non-conserved AID residues, in addition to the conserved residues in G $\beta\gamma$ binding.

When comparing the binding of G $\beta\gamma$ to the mutant AID_A-GST fusion proteins to the wild-type AID_A-GST fusion protein (Fig. 3d), G $\beta\gamma$ binding was lost upon mutation of Q1, Q2, L7, G9 and Y10. The binding was, however, maintained for mutants of E₄, W₁₃, I₁₄ and E₁₈. These data remain consistent with the identification of Y-WI (10–14) as being critical for the binding of the Ca²⁺-channel β -subunit⁹. Y10 could possibly be a common interactive residue for both the Ca²⁺-channel β -subunit and the G $\beta\gamma$ complex, perhaps being responsible for some of the functional antagonism observed between activated G proteins and the Ca²⁺-channel β -subunit^{16,18}. Noticeably, the N-terminal part of the AID region of the three interacting α_1 subunits contains a motif (Q-ER) (1–5) that was previously identified in a G $\beta\gamma$ -interacting type of adenylyl cyclase and is necessary for its regulation by the G $\beta\gamma$ complex¹⁹. In this motif, Q1 and E4 are conserved in all α_1 subunits, while R5 is present only in the G $\beta\gamma$ -interacting α_1 subunits (A, B and E). The importance of Q1 and R5 is demonstrated in Fig. 3d, although E4 seemed not to be as essential as in the case of adenylyl cyclase (a similar result was obtained when E4 is substituted by a serine; data not shown). Overall, these results define QQ-ER-L-GY (1–10) as an essential motif for G $\beta\gamma$ binding to the AID site in voltage-dependent Ca²⁺ channels. The involvement of additional AID residues in the interaction can not be excluded, however, as not all AID residues were tested.

A deletion approach to determine the D2 sequence responsible for the binding of G $\beta\gamma$ demonstrated that several stretches of the D2_A sequence (401–435, 401–461, 436–487 and 450–487) were all capable of interacting with G $\beta\gamma$ (data not shown), suggesting that the D2 binding domain could not be localized further, and may be comprised of several microsites.

Next, we analysed the functional importance of G-protein bind-

ing to the I-II cytoplasmic linker. It is well established that activation of G proteins leads to several biophysical changes in the gating of voltage-dependent Ca²⁺ channels through a membrane-delimited²⁰ and voltage-dependent²¹ pathway that does not involve a second messenger system²². Injection of cRNAs encoding the α_{1A} and β_4 subunits^{10,23} in *Xenopus laevis* oocytes results in the expression of functional voltage-dependent Ca²⁺ channels (Fig. 4a). A membrane depolarization to 0 mV triggers a maximum inward current of $-1,831 \pm 893$ nA ($n = 5$) that peaks at 11.1 ± 1.9 ms ($n = 15$) after the start of the pulse (Fig. 4a, left). Irreversible activation of several endogenous²⁴ G proteins (G_o, G_i and G_s), by injection of 200–500 μM of GTP- γS in *Xenopus* oocytes, bypassing receptor activation, results in a significant slowing of the activation process. This slowing can be measured by a delayed latency of the peak current that now occurs 26.4 ± 2.3 ms ($n = 4$) after the start of the depolarization (Fig. 4a, right). The effect of GTP- γS can be reversed by a strong depolarizing prepulse (data not shown). Slowing of the activation kinetics has been interpreted as the gradual and partial dissociation of activated G protein from the channel in its open state⁶, although a conformational change producing a slower gating cannot be ruled out⁵. The rather slow time course of GTP- γS action (maximum effect 8 ± 2 min after injection) corresponds well with the slow activation of G proteins by GTP analogues in the absence of receptor agonists²⁵. Activation of G proteins had a very limited effect on current amplitude itself, with an average reduction of $12.4 \pm 0.1\%$ ($n = 4$), which suggests that the effects of G proteins on the current kinetics and amplitude can be independent of each other.

Expression of the mutant α_{1A} R387E subunit (substitution of the R residue of the G $\beta\gamma$ interacting motif (QQ-ER-L-GY) (1–10)) along with β_4 results in a current that activates at a rate similar to that of the control channel, with a time to peak of 13.6 ± 2.4 ms

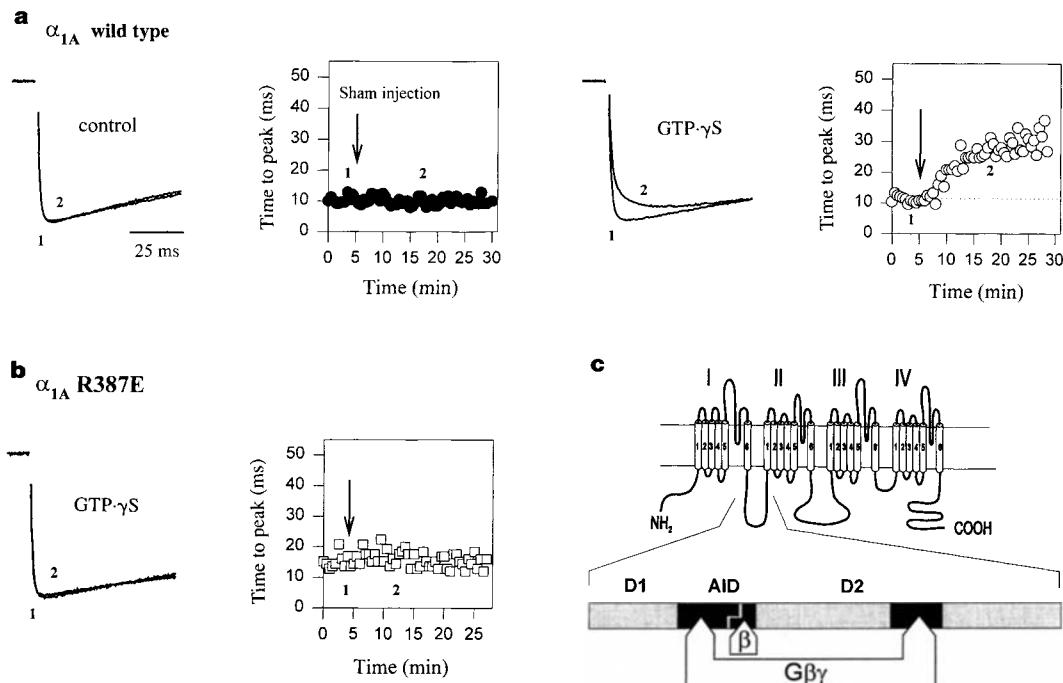


Figure 4 Effect of activated G proteins on the activation kinetics of the wild type and the mutant α_{1A} R387E channel (AID R5E). **a**, Left, no effect of sham injection on activation of wild-type α_{1A} channel. Right, slowing of activation kinetics of wild-type α_{1A} channel by injection of 300 μ M GTP- γ S (indicated by arrow). Currents were normalized to each other to illustrate the slowing of current activation. **b**,

Absence of effect of GTP- γ S on the activation time course of the α_{1A} R387E mutant channel. Addition of 300 μ M GTP- γ S indicated by arrow. Peak current amplitudes are -2.8 μ A (**a**, left), -2.9 μ A (**a**, right) and -2 μ A (**b**). **c**, Schematic representation of the G $\beta\gamma$ interaction site on the I-II cytoplasmic linker of the α_1 subunit.

($n = 7$) at 0 mV. In contrast to the wild-type α_{1A} channel, the time to peak of the mutant α_{1A} R387E channel was not regulated by the injection of GTP- γ S into the cell ($n = 5$; Fig. 4b), whereas two chimaeras in which the entire AID_A sequence was replaced by AID_B or AID_E could still be regulated (data not shown). These results emphasize the importance of this arginine residue, not only in binding of G $\beta\gamma$ to the AID region (Fig. 3d), but also for regulation of channel activity. Mutation of this arginine residue does not affect the full-length I-II_A loop binding to G $\beta\gamma$ (Fig. 3c) or to the Ca²⁺ channel β -subunit (data not shown). The fact that most of the G-protein regulation can be blocked by this AID mutation also suggests that the D2 site attachment is not sufficient for G-protein modulation, or at least for its kinetic effect.

We have shown that the G $\beta\gamma$ complex interacts directly with three types of voltage-dependent Ca²⁺ channels (α_{1A} , α_{1B} and α_{1E}), which is consistent with early observations that G-protein regulation is membrane delimited and voltage dependent. These channels therefore represent a second class of ion channels, in addition to the inwardly rectifying potassium channel^{26,27}, that can be regulated directly by the G $\beta\gamma$ complex. The binding site of this complex is localized within the cytoplasmic linker that connects the hydrophobic repeats I and II of these α_1 subunits (Fig. 4c), and mediates G-protein modulation, Ca²⁺-channel β -subunit stimulation, and protein kinase C upregulation^{8,28}. Similar to the GIRK1 potassium channel²⁷, the binding site comprises two regions, the AID region and the D2 region. The AID sequence is required for channel regulation, whereas it is possible that the D2 sequence positions the G $\beta\gamma$ close to the α_1 channel. The proximity of the G-protein binding site to the Ca²⁺-channel β -subunit interaction site on AID probably represents the structural basis for some of the functional antagonism (current amplitude) observed between these molecules^{16,18}. The proximity of the G $\beta\gamma$ binding site to the AID motif means that it will be interesting to determine whether the binding of the Ca²⁺-channel β -subunit may allosterically modify the regulatory effect of G $\beta\gamma$ complex. □

Methods

Preparation of fusion proteins. DNA constructs for GST fusion proteins of the α_{1A} subunit (BI-2 clone¹⁰) were constructed by subcloning base pairs 1080–1459 (I–II–GST), 2633–3201 (II–III–GST²⁹), 1080–1199 (D1–AID–GST), 1080–1145 (D1), 1146–1460 (AID–D2–GST) and 1200–1460 (D2–GST) into pGEX 2TK or pGEX KG vectors. GST fusion proteins of the full-length I–II linkers were expressed from base pairs 1228–1521 (I–II_S–GST, amino acids 334–432), 1066–1449 (I–II_B–GST, amino acids 356–483), 1303–1662 (I–II_C–GST, amino acids 435–554) and 903–1281 (I–II_E–GST, amino acids 302–427) of rabbit α_{1S} , rat α_{1B} , rabbit α_{1C-a} and rat α_{1E} . The AID_A–GST fusion protein expresses AID_A and 14 N- and 18 C-terminal sequences of D1_A and D2_A, respectively (AID_A–GST)⁸. Mutations of AID residues were prepared as described previously⁸. Mutant AID_A R5E was constructed by cassette mutagenesis using the mutagenic primer 5'-CAGCAGCAGATTGAA-GAGGAGCTCAACGGGTAC-3'. The D2_A–MBP fusion-protein construct was prepared by subcloning base pairs 1200–1460 of the α_{1A} into pMal-c2 vector. Fusion-protein constructs of the truncated D2_A segment contain base pairs 1200–1304 (401–435 of D2), 1200–1382 (401–461 of D2), 1305–1460 (435–487 of D2) and 1347–1460 (450–487 of D2). The fusion proteins were sequenced and purified according to standard procedures¹⁷.

Binding assays. Full-length G α_0 , G β_1 and G γ_2 cDNA sequences were subcloned into pcDNA3 vector. The ³⁵S-labelled G-protein α_0 , γ_2 and $\beta_1\gamma_2$ probes were synthesized *in vitro*³⁰ by coupled transcription and translation (TNT system, Promega). Free ³⁵S-methionine was removed with a PD10 column (Pharmacia) from the radiolabelled proteins for all binding experiments. Binding of the G-protein probes was performed by coupling equal amounts of the fusion proteins to glutathione-sepharose beads, and incubating with 0.5–2.0 pM of probes overnight at 4 °C in buffer consisting of (in mM) 10 HEPES, pH 7.4, 1 DTT, 1 EDTA, 150 NaCl, 0.1% CHAPS, 4 mg ml⁻¹ BSA. The G α_0 subunit was activated by addition of 100 μ M GTP- γ S in the binding buffer. The beads were washed on ice with 4 ml 0.05% CHAPS in PBS. The beads were then loaded on SDS-PAGE or subject to scintillation counting. Rat brain membranes were solubilized for 1 h at 4 °C in buffer containing (in mM) 50 Tris, pH 7.4, 1 DTT, 1 EDTA, 1,000 NaCl, 1.5% CHAPS, with a cocktail of protease inhibitors (Complete, Boehringer Mannheim). Insoluble material was

removed by centrifugation at 100,000 r.p.m. for 12 min in a TL100.3 rotor (Beckman). The supernatant was diluted 10-fold with buffer (in mM): 20 Tris, pH 7.4, 1 DTT, 1 EDTA, 1 GDP, 10 NaF, 10 MgCl₂, 0.05 AlCl₃, with the protease inhibitor cocktail. Fusion proteins 5–20 µg were added to 25 ml of diluted brain extract, incubated at 4 °C overnight and washed as described above. Proteins analysed on SDS–PAGE (16% gel) were either dried and exposed to autoradiogram film (X-OMAT AR, Kodak) or transferred to nitrocellulose for western blot analysis. The D2_A–MBP protein was used for dose–response analysis because it purified better than the GST–D2_A fusion protein. The saturation curve was generated as previously described¹⁷.

Electrophysiological recordings. Complementary RNAs were transcribed *in vitro* using the SP6 RNA polymerase with the rabbit brain α_{1A} plasmid and T7 RNA polymerase with rat brain β₄ plasmid. Wild-type, mutant or chimaeric α_{1A} (0.4 µg µl⁻¹) were co-injected with 0.2 µg µl⁻¹ β₄ cRNA into stage V or VI *Xenopus* oocytes. Ba²⁺ currents were recorded¹⁷ using the following bath solution (in mM): 10 Ba(OH)₂, 80 NaOH, 2 KCl, 1 niflumic acid, 0.05 EGTA, 5 HEPES, pH 7.4, adjusted with methanesulphonic acid. The membrane potential was held at -90 mV and transiently pulsed at 0 mV for 500 ms every 30 s. Injection of GTP-γS was performed 5–6 min after control run-up was achieved.

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Traction forces of cytokinesis measured with optically modified elastic substrata

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Animal cells dividing in culture undergo a dramatic sequence of morphological changes, characterized by cytoskeletal disassembly as cells round up, redistribution of actin, myosins and other cytoplasmic and surface molecules into the cleavage furrow^{1–11},

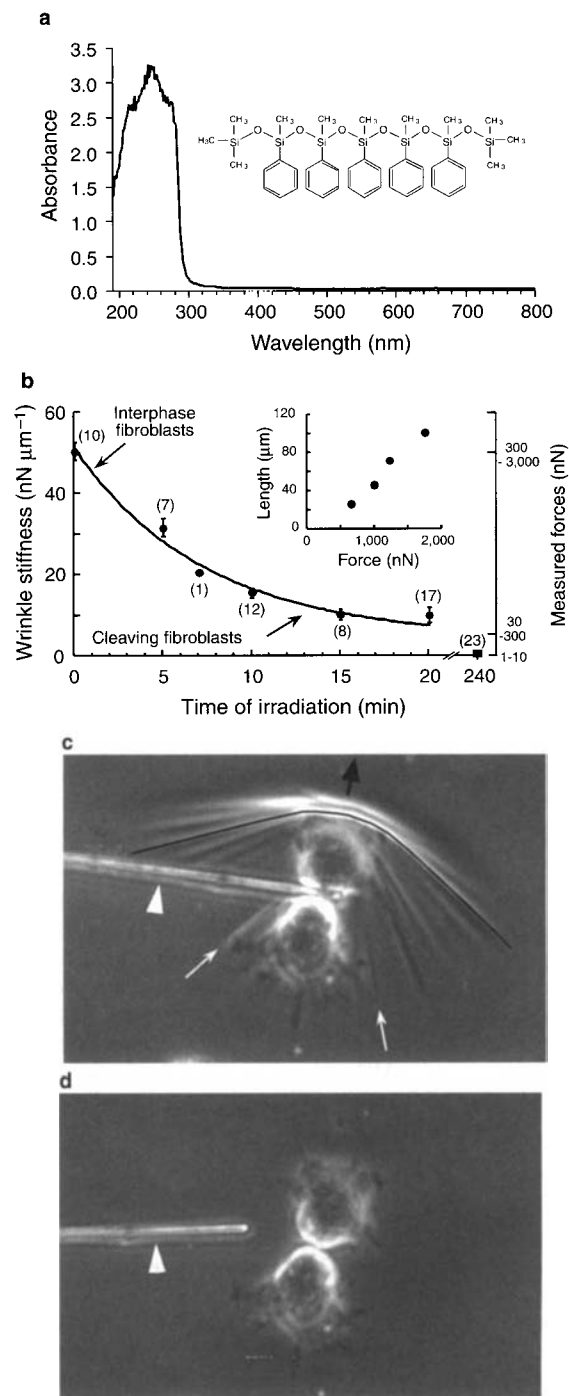


Figure 1 Fabrication and calibration of silicone sheets. **a**, Structure and absorbance spectrum of the silicone fluid. **b**, Stiffness of silicone substrata as a function of ultraviolet irradiation. Points indicate mean ± s.e.m.; number of wrinkles is given in parentheses. Calibration images were acquired from about 5 positions per sheet, and the results of measurements from 15 sheets are shown. Stiffness fell nearly exponentially with irradiation over 0–20 min according to the formula: stiffness = 46.3 × exp(–0.134 × t) + 4.4, where t is the time (min). Stiffness at 240 min of irradiation was 0.26 ± 0.02 nN µm⁻¹. The arrows on the graph indicate the approximate substratum stiffness used to measure forces produced by interphase and dividing fibroblasts in the ranges indicated on the right vertical axis. The inset shows the relation between wrinkle length (half the length of compression wrinkles) and force for the calibration from which the images in **c** and **d** were taken. **c**, **d**, Calibration of silicone-sheet compliance. Images of a pair of fixed cells shown during (**c**) and after (**d**) application of force by a calibrated microneedle (white arrowhead). Phase-contrast microscopy; images are 147 µm wide. In **c**, the direction of applied force is indicated by a black arrow; the black line traces the compression wrinkle, and white arrows point to tension wrinkles (see text).

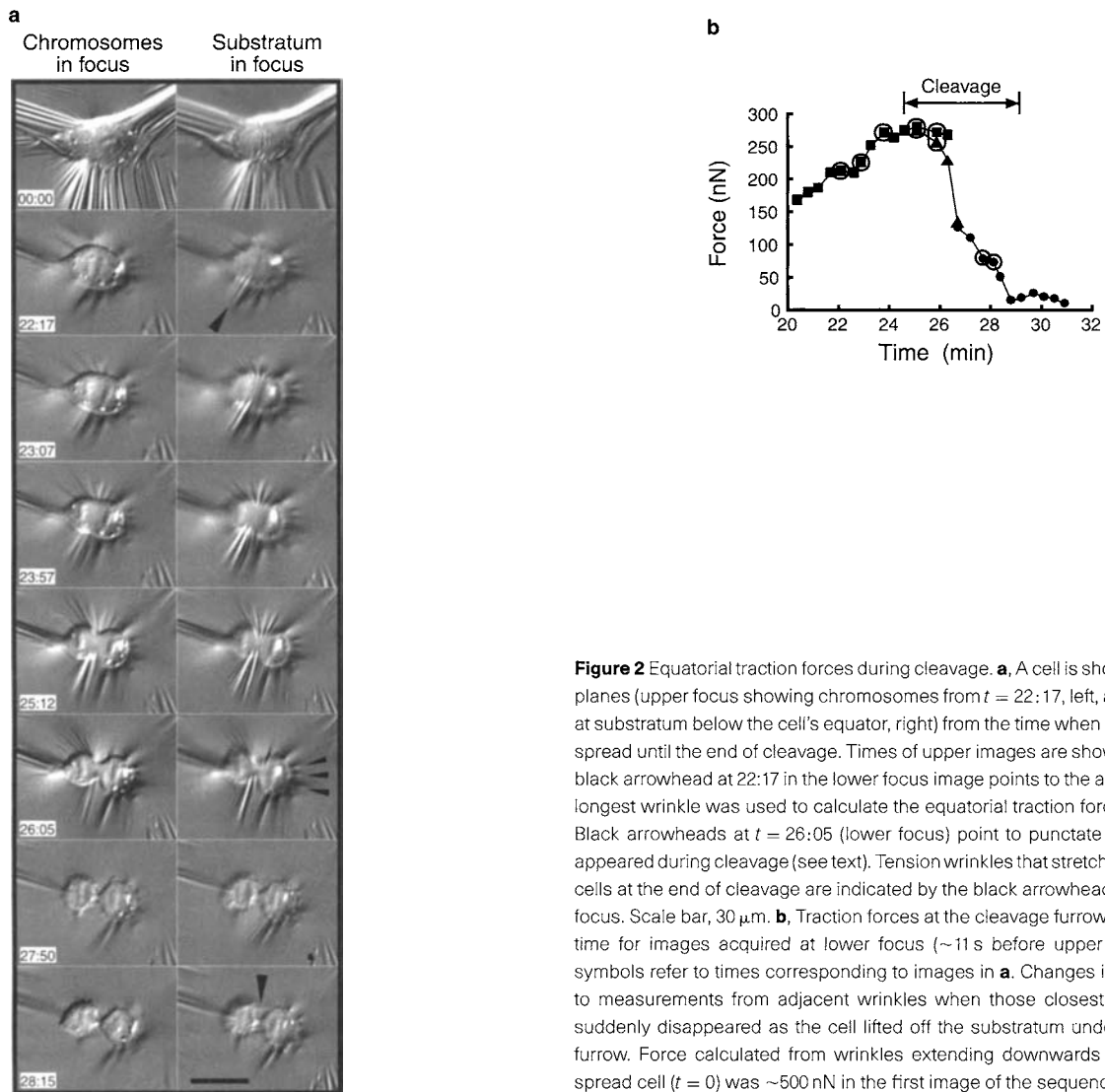


Figure 2 Equatorial traction forces during cleavage. **a**, A cell is shown at two focal planes (upper focus showing chromosomes from $t = 22:17$, left, and lower focus at substratum below the cell's equator, right) from the time when it was still partly spread until the end of cleavage. Times of upper images are shown (min:s). The black arrowhead at 22:17 in the lower focus image points to the area in which the longest wrinkle was used to calculate the equatorial traction forces shown in **b**. Black arrowheads at $t = 26:05$ (lower focus) point to punctate distortions that appeared during cleavage (see text). Tension wrinkles that stretched between the cells at the end of cleavage are indicated by the black arrowhead at 28:15, lower focus. Scale bar, 30 μm . **b**, Traction forces at the cleavage furrow plotted against time for images acquired at lower focus (~ 11 s before upper focus). Circled symbols refer to times corresponding to images in **a**. Changes in symbols refer to measurements from adjacent wrinkles when those closest to the equator suddenly disappeared as the cell lifted off the substratum under the cleavage furrow. Force calculated from wrinkles extending downwards from the partly spread cell ($t = 0$) was ~ 500 nN in the first image of the sequence.

and respreading¹², before daughter cells finally separate at the mid-body¹³. Knowledge of forces governing these movements is critical to understanding their mechanisms, including whether formation of the cleavage furrow results from increased force generation at the equator^{14,15} or relaxation at the poles¹⁶, and whether traction force subsequently mediates cytofission of the intercellular bridge^{5,13,17,18}. We have quantitatively mapped traction forces in dividing cells, by extending the silicone-rubber substratum method¹⁹ to detect forces of nanonewtons to micronewtons. We used a new silicone polymer to fabricate substrata whose compliance can be adjusted precisely by ultraviolet irradiation. We show that traction force appears locally at the furrow in the absence of relaxation at the poles during cleavage. Force also rises as connected daughter cells respread and attempt to separate, suggesting that tension contributes to the severing of the intercellular bridge when cytokinesis is completed.

We have measured traction forces at the cleavage furrow and elsewhere on the surface of fibroblasts adherent to a substratum during cell division. Although traction force has been observed to fall to below the level of detection as fibroblasts round up before the cleavage furrow is formed¹⁵, and cleavage forces have been estimated from the deflection of needles inserted into echinoderm eggs^{20,21}, force has not been measured in adherent mammalian cells during cytokinesis. To measure such weak forces in a non-invasive manner, we developed a method of using ultraviolet irradiation to increase the compliance of transparent elastic substrata fabricated from a

new silicone polymer (Fig. 1). Compliance was adjusted so that cells generated wrinkles of measurable length without large movements in the substratum; wrinkle length was about 20 times larger than displacement of wrinkles and other markers in the rubber sheet. Movements were therefore no more than about 3 μm at the cell, thus providing for nearly isometric force measurements.

Traction forces were detected at the furrow in five cells that adhered at their equator to highly compliant silicone sheets. A time-lapse sequence of one of the dividing cells, from the time it was rounding up to the time of cleavage-furrow formation, is shown in Fig. 2a. While the cell was still partly spread and generating high traction forces, long wrinkles extended outside the field of view (Fig. 2a, $t = 0$). As the cell rounded up, most wrinkles were lost, and force calculated from the lengths of the wrinkles (Fig. 1; see Methods) decreased from a few hundred to a few tens of nanonewtons (Fig. 2a, black arrowhead at $t = 22:17$; Fig. 2b). Wrinkles disappeared altogether from many areas of the cell, indicating that traction forces were below the 10–20 nN that could be reliably measured using this sheet (stiffness $\sim 10 \text{ nN } \mu\text{m}^{-1}$ wrinkle length; see Methods). Traction force increased transiently near the furrow during cleavage, as shown by the appearance of curved wrinkles parallel to the equator (shown at times 23:07 to 26:05 in Fig. 2a). The curvature of the wrinkles ($t = 25:12$) shows that the substratum was compressed towards the centre of the cleavage furrow (see Methods), although force was nearly isometric as movement of the wrinkles was only $\sim 1 \mu\text{m}$. Such equatorial forces could be transmitted from

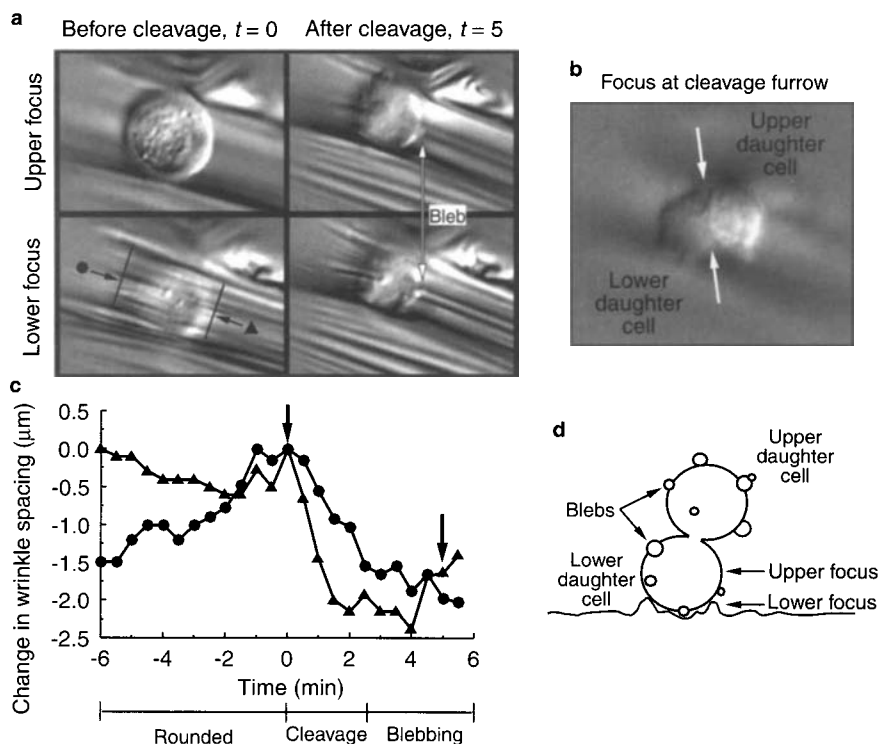


Figure 3 Polar traction forces during cleavage. The cell adhered near its pole to the silicone sheet and divided approximately parallel to the substratum, as indicated in **d**. Images in **a** were focused on the substratum (lower focus) and on the middle of the adherent cell (upper focus) before and after cleavage (at times indicated by arrows in **c**). The image in **b** shows the cleavage furrow (arrows, $t = 7.5$ min); the daughter cells were partly out of focus above and below the focal plane. In **a**, a wrinkle that extended from a bleb is indicated by the double-headed

arrow in the images at $t = 5$ min. Spacing between wrinkles bracketing the cell was measured at two positions indicated by lines to the left and right of the cell in the lower focus image at $t = 0$. The lines are drawn between wrinkles whose positions were tracked before and during cleavage; changes in the distance between these wrinkles are shown in **c** (circles and triangles refer to spacings at left and right of cell, respectively). In **a**, the line to the left of the cell indicates $19 \mu\text{m}$.

a simple 'contractile ring' through the cortex to the cell surface^{6,11} to produce traction, but cytoskeletal filaments recently observed at an angle to the furrow^{8,22,23} may also contribute contractile activity or provide a directed path for propagation of force from the furrow to sites of substratum adhesion. Such forces transmitted through retraction fibres may have been responsible for the punctate distortions that were often visible around the periphery of rounded cells during cleavage (Fig. 2a, $t = 22:17$ to $28:15$; black arrowheads at $t = 26:05$), but higher-resolution images at several focal planes will be required to test this possibility.

Wrinkles parallel to the equator began to disappear when constriction of the cell was about 50% complete, owing in part to detachment of the cell from the substratum under the cleavage furrow (judged in Fig. 2a by the disappearance of the intercellular bridge from the focal plane of the substratum). When cleavage was nearly complete, this cell exhibited a reversal of traction from compression to extension below the furrow, causing wrinkles to stretch between the two daughter cells (Fig. 2a at $t = 28:15$). This behaviour was observed on several occasions, and was associated with widening of the furrow. An analogous reversal of the movement of cytoskeletal fibres in the furrow region has also been observed using fluorescent analogues of myosin II incorporated into the cytoskeleton⁸. This change in direction of traction force and cytoskeletal movement implies a mechanism of cleavage in which constriction at the equator initially deepens the furrow, and subsequent widening of the furrow in adherent cells is accomplished by cortical contractility similar to tail retraction in freely migrating fibroblasts. The latter process is dependent on traction¹, and could not occur in non-adherent cells, including isolated eggs. A general similarity in the force-generating mechanisms of cleavage and cell motility has been suggested^{8,23,24}, and the relative extents to which

equatorial constriction and cortical retraction control cleavage undoubtedly depends on the type of cell and its circumstances.

Polar-relaxation models of cleavage predict that tension in the polar cortex will decrease, accompanied by cortical movement from the pole towards the equator; spacing between wrinkles bracketing the pole should therefore increase¹⁶. As a test of this proposal, we monitored two cells that adhered to the substratum through one pole, and measured spacing between wrinkles during formation of the cleavage furrow. In the example shown in Fig. 3, the cleavage furrow was approximately parallel to the substratum and the chromosomes formed a circular array in the image plane. The symmetrical arrangement of wrinkles about the pole, and the slight curvature of those wrinkles at the edges of the cell, suggest that the substratum was compressed toward the pole, with net force directed perpendicular to the wrinkles. Cleavage was inferred from a decrease in cell diameter during furrowing, and also by the appearance of an 'upper' daughter cell (Fig. 3; separate image at higher focal plane shows cleavage furrow (white arrows)). The graph in Fig. 3 shows that before cleavage there were small increases and decreases in wrinkle spacing (about $-0.5 \mu\text{m}$ to $+1.5 \mu\text{m}$), but that during cleavage there was a more significant decrease in spacing ($\sim 2 \mu\text{m}$ over 4 min), opposite to the change expected for polar relaxation. Additional evidence that force was directed towards the pole was provided by wrinkles at the edges of the cell; spacing between these wrinkles increased when they were released as cell diameter fell during cleavage (for example, right-hand spacing in Fig. 3, after $t = 4$ min). We suggest that these results are a direct test of polar-relaxation models, and show that cleavage does not require polar relaxation *per se* in fibroblasts, a conclusion consistent with other evidence in echinoderm eggs¹⁴.

A complete description of cortical forces and movements would

require improvements in methodology to allow simultaneous measurements in three dimensions over the entire surface of a single cell¹⁵. For example, our results do not exclude relaxation of the non-adherent pole or areas between the pole and equator. We also note that cortical movements in response to force at the equator could dissipate away from the furrow if cortical stiffness were lower in the equatorial region than elsewhere, consistent with studies showing movements of lectin receptors and myosin II largely confined to areas between the equator and about halfway to the poles^{8,11}.

Daughter cells were not completely separated by the cleavage furrow. Instead there followed an interval lasting from several minutes to over an hour during which there was vigorous motile activity of several kinds, including protrusion of blebs, filopodia and lamellipodia, with eventual respreading and migration of daughter cells that remained connected at the mid-body. Traction forces produced by many protrusive structures, including blebs (Fig. 3, double-headed arrow), were detected on the highly compliant

substrata; it was therefore possible to map patterns of wrinkles to test the possibility that traction forces contribute to respreading and ultimate separation of cells during cytokinesis. A sequence of images is shown in Fig. 4, in which it can be seen that, during respreading, straight wrinkles extending from the lamellum were as large or larger than those present elsewhere under the cells (400–1,200 nN for the right cell, and 300–500 nN for the left cell, during the time period 19:40 to 35:22), suggesting that forces acted to pull the edge of the cell forwards as it flattened. Significant force was applied by the leading edges in all cells examined.

Final separation of daughter cells involved migration and severing of the intercellular connection¹³. This was true of cells on compliant or stiff silicone sheets and of those on glass. If traction force facilitates separation, then force should rise until the intercellular bridge ruptures, and the substratum should be compressed between the daughter cells. In the example of Fig. 4, the number and average length of wrinkles increased substantially until the cells separated, at which time the increase in wrinkling was partly reversed as the substratum recoiled and force fell rapidly to the level present during free migration. The recoil moved the cells approximately along their axes of migration, indicating that the silicone sheet had been compressed in that direction. In many fibroblasts, the furrow became less distinct as cells respread following cleavage, and it appeared as if the sides of the furrow came together. The cell in Fig. 4 shows an example of this process in which a broad area of contact formed before narrowing as daughter cells migrated apart (compare $t = 8:20$ with $t = 19:40$ to $30:45$). A strong correlation was observed between a rise of traction force before cell separation and the apparent degree of contact between connected daughter cells. Force rose appreciably during separation in four of six cells monitored throughout cell separation, and in the two exceptions the furrow did not close and the intercellular connection remained quite thin (diameter <10% cell diameter). In several additional experiments, cells with broad areas of contact generated large forces for extended periods (15–60 min) as they migrated in an apparent attempt to separate.

A contribution of traction force to daughter-cell 'fission' had been suggested to complement formation of the cleavage furrow in dividing amoebae (see refs 5 and 17 for discussion); evidence for 'traction-mediated cytofission' has come from mutant amoebae devoid of conventional myosin^{5,18}. The relative importance of these two mechanisms will vary with cell type and motile activity¹⁷, and structural changes under biochemical control are also important^{13,25}. However, our direct measurements of traction forces suggest that mechanical tension can contribute to thinning and eventual breakage of the intercellular bridge, thus constituting an important mechanism of cytokinesis in cells attached to a substratum. □

Methods

Cell analysis. Swiss 3T3 fibroblasts (ATCC) were seeded onto silicone sheets in DMEM + 10% calf serum (passages 118–125) and cultured at 37°C. Coverslips with silicone sheets were immersed rapidly into culture medium because slow wetting resulted in destruction of the sheet owing to surface tension of the hydrophobic silicone²⁶. The coverslips were placed in a temperature-controlled chamber (37°C) mounted on an Zeiss Axiovert microscope. VEC-DIC and phase-contrast images were acquired with a heated objective onto a video camera (C2400 Newvicon, Hamamatsu) using objective magnifications of $\times 40$ (1.3 NA) and $\times 100$ (1.3 NA). Images were analysed using STC View and NIH Image software.

Silicone polymer. The silicone polymer used to fabricate the elastic substrata was phenylmethyl polysiloxane (Fig. 1a), trimethyl terminated (Dow Corning 710 fluid). It is traditionally used for high-temperature applications such as diffusion pump oil. The phenyl groups confer two critical optical properties. First, the silicone absorbs strongly in the far ultraviolet range ($\lambda = 254$ nm; Fig. 1a), and this high-energy irradiation weakens the silicone sheet and thus increases its compliance in a precisely controllable way. This presumably occurs

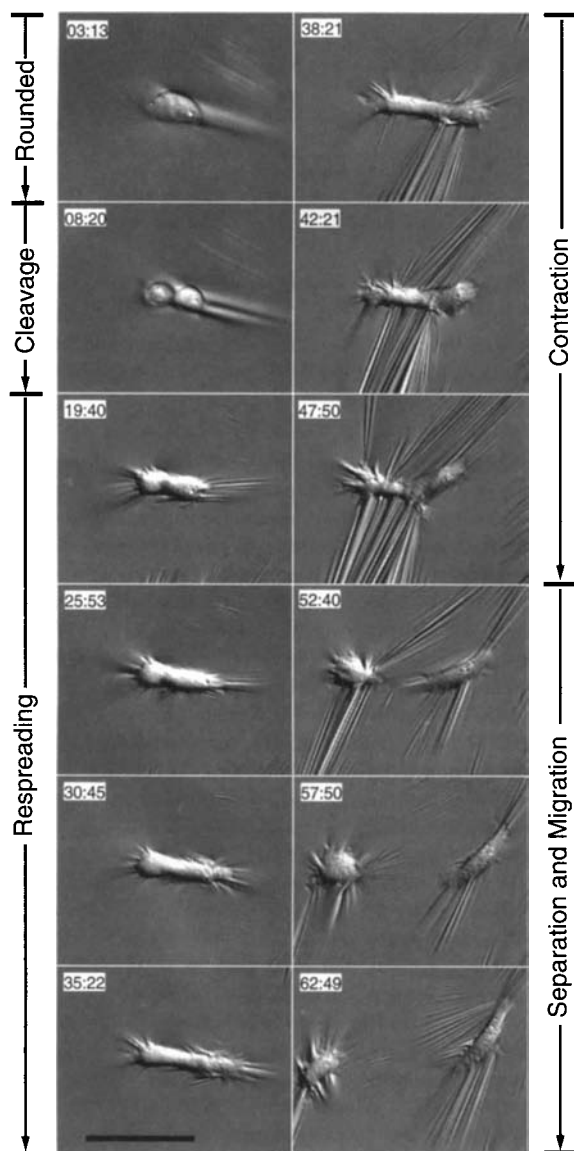


Figure 4 Traction forces during daughter-cell respreading and separation. Images of a cell before cleavage to the time of daughter-cell separation and migration. The daughter cells disconnected and retracted their tails between $t = 51:40$ and $t = 52:40$, and the resulting recoil of the elastic sheet caused a rapid separation of the cells. Scale bar, 80 μ m.

by disrupting either the silicone polymer itself or crosslinks formed between polymers during vulcanization (see below). The compliance of the traditional silicone (dimethyl polysiloxane) is not noticeably increased by similar irradiation. The second optical property is a high index of refraction (1.536), similar to glass, and so is suited to many types of microscopy, including interference reflection microscopy (IRM). IRM yielded low-contrast images with the previous silicone, and is useful because it allows visualization of cell-substratum contacts at which traction force is applied.

Creation of thin films. Thin films of silicone rubber were produced by vulcanizing the surface of a layer of fluid in one of two ways. The first method was to pass a coverslip bearing silicone fluid through a Bunsen-burner flame for about 1 s on a motorized mount, similar to the original method²⁷, but the sheets are not easily reproduced and are somewhat heterogeneous. We have overcome this limitation by developing a new method in which heat is applied in a controllable manner by a hot tungsten wire (K.B. and D.L.T., manuscript in preparation). This technique also allows the fabrication of stiffer sheets than is possible with flame vulcanization, and should complement work in which crosslinking of the silicone has been controlled by the use of glow discharge²⁸.

Measurement of forces. Forces applied by cells to silicone sheets were estimated by using flexible microneedles (white arrowheads in Fig. 1c, d) to calibrate sheets produced in the same way. Needle stiffness was measured by hanging weights fabricated from small glass beads. To apply force to sheets in a reversible manner without causing damage, we fixed cells to sheets (1% glutaraldehyde for 10 min) and pushed on the fixed cells with needles (Fig. 1c). The applied force produced wrinkles that were completely reversible (Fig. 1c, d), and most disappeared within a fraction of a second of the stress being removed. Wrinkle 'stiffness', calculated by dividing applied force by wrinkle length, was approximately linear (Fig. 1b, inset). We have not expressed force in terms of stress, as this requires estimates of the area through which force is transmitted; this type of study will be reported separately using IRM²⁹. Compliance was increased by short-wave ultraviolet irradiation (6 W at 15–20 mm above the sheet, $\lambda = 254$ nm; 300 μ W cm⁻² at 15 cm from the surface (manufacturer's specification), model UVGL-58, UVP Inc.). Ultraviolet illumination caused wrinkle stiffness to fall exponentially (Fig. 1b). The stiffness of sheets was usually between 10 and 50 nN μ m⁻¹, although much more compliant sheets are easily produced by prolonging the ultraviolet treatment (0.26 nN μ m⁻¹; Fig. 1b).

Wrinkles in a silicone sheet are of two general types^{27,30}: curved 'compression' wrinkles centred on, and orientated perpendicular to, the applied force (black line on wrinkle in Fig. 1c where the sheet was compressed); and straight 'tension' wrinkles originating near, and oriented parallel to, the applied force (white arrows in Fig. 1c where the sheet was stretched). The wrinkles in Fig. 1c show that there is continuity between these two extremes. 'Stiffness' of the two types of wrinkles was found to be more consistent using half the length of compression wrinkles (Fig. 1b, inset). Movement of markers in the plane of the substratum is an alternative measure of strain^{15,27,28,31} and, because this allows calculation of 'traction fields' over an area^{30,31}, a chimaeric method using both types of strain might be valuable. However, wrinkles afford some advantages over markers. (1) For a given stress, wrinkle length is about 20 $\times$ the displacement of markers (such as cells and debris), thus providing higher signal-to-noise ratio and allowing more nearly isometric force measurements. (2) Absolute force can be measured at any instant, and comparison to marker positions at a separate time is not required, thus avoiding movement artefact. (3) The location and magnitude of traction force can be obtained from the position of a single wrinkle, and need not be estimated from the displacements of many markers at finite spatial resolution.

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HOX11 interacts with protein phosphatases PP2A and PP1 and disrupts a G2/M cell-cycle checkpoint

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Hox11 is an orphan homeobox gene that controls the genesis of the spleen¹. *HOX11* is also oncogenic, having been isolated from a chromosomal breakpoint in human T-cell leukaemia^{2–4}. Transgenic mice that redirected *HOX11* to the thymus demonstrated cell-cycle aberration and progression to malignancy⁵. We observed that the protein *HOX11* interacted with protein serine-threonine phosphatase 2A catalytic subunit (PP2AC), as well as protein phosphatase 1 (PP1C) in mammalian cells. Inhibition of PP2A can regulate the cell cycle and control the activation of maturation-promoting factor in *Xenopus* oocytes⁶. Microinjection of *HOX11* into *Xenopus* oocytes arrested at the G2 phase of the cell cycle promoted progression to the M phase. G2 arrest can be induced by γ -irradiation, but is eliminated by expression of *HOX11* within a T-cell line. Thus *HOX11* is a cellular oncogene that targets PP2A and PP1, both of which are targets for oncogenic viruses and chemical tumour promoters^{7,8}. This interaction suggests a mechanism by which a homeobox can alter the cell cycle.

Many transcription factors form oligomeric complexes, prompting a search for proteins that interact with *HOX11*. We used the yeast two-hybrid system⁹ and *HOX11* (amino acids 1–211) to screen a murine T-cell cDNA library. Both the PP2AC- α and PP2AC- β catalytic subunits were isolated. Deletion mutants of *HOX11* were tested against PP2AC- β to further restrict the interaction domain (Fig. 1). A minimal region of *HOX11* (amino acids 149–190) outside the homeodomain was capable of binding PP2AC. To confirm this interaction, PP2AC derived from Jurkat cell lysates was shown to bind to recombinant glutathione S-transferase (GST)–*HOX11* amino-terminal, but not to *HOX11* carboxy-terminal, protein (Fig. 2a). In the same set of experiments, PP1C was also shown to bind *HOX11* (Fig. 2a). These results suggest that *HOX11* binds directly to the catalytic subunits of these proteins. This may reflect the known homology between PP2AC

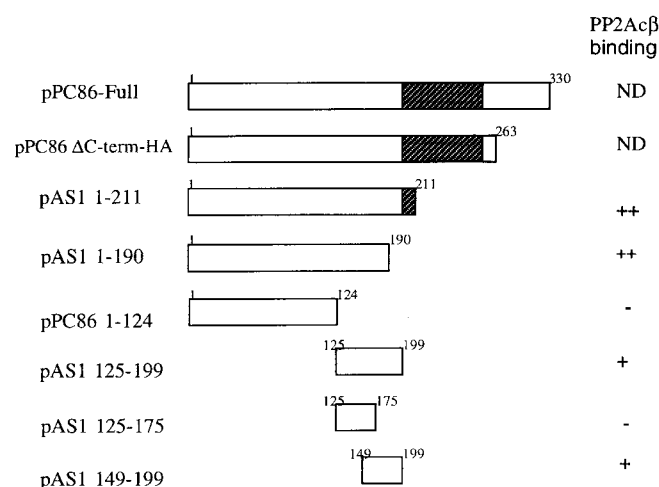


Figure 1 HOX11 interaction with PP2A by yeast two-hybrid analysis. PP2Ac-β binding to HOX11 deletion mutants: ++, strong; +, weak; -, no detectable β-galactosidase activity. Numbers correspond to the amino-acid position within HOX11. The homeobox is shaded. Constructs pPC86-Full and pPC86ΔC-term-HA possessed inherent transcriptional activation in yeast two-hybrid analysis and were not used.

and PP1C, a structural basis that also resulted in cross-reacting antibodies¹⁰. We generated an anti-HOX11 monoclonal antibody (1D7) against recombinant HOX11 (amino acids 261–330) that efficiently immunoprecipitated HOX11 from the t(10;14)-bearing acute lymphoblastic leukaemia (ALL)-Sil T-cell leukaemia line, but not from Jurkat cells that do not express *HOX11* (Fig. 2b). Western blot analysis of HOX11 immunoprecipitates showed that PP2AC and PP1C had been co-immunoprecipitated with the HOX11 from ALL-Sil (Fig. 2c, d). This demonstrates that HOX11 physically interacts with both PP2A and PP1 within mammalian cells.

To assess the functional consequence of the interaction between HOX11 and PP2A, we examined the effects of HOX11 on germinal vesicle breakdown (GVBD) in *Xenopus* oocytes, which occurs in M phase. PP2A and perhaps PP1 play a role in maintaining the G2 meiotic arrest of *Xenopus* oocytes by preventing activation of maturation-promoting factor (MPF)^{11,12}. Injection of okadaic acid, an inhibitor of PP2A and PP1, promoted nearly uniform GVBD (Fig. 3f), as previously described¹³. Injection of *HOX11*ΔC-terminus-HA (haemagglutinin tag) sense RNA resulted in GVBD in approximately 65% of oocytes (Fig. 3a). We used a closely related family member (*HOX11L1*)¹⁴, which does not bind PP2A, to generate a control protein. A chimaeric HOX11L1/HOX11-HA molecule, in which the N-terminal portion of HOX11L1 (amino acids 1–145) was substituted for HOX11 (amino acids 1–199), did not stimulate GVBD; neither did anti-sense RNA or HOX11 (amino acids 1–124) (Fig. 3a). HOX11ΔC-terminus-HA, but not the control injections, resulted in histone H1 kinase activity, confirming the G2/M transition (Fig. 3e). PP2AC of injected frog cell lysates was co-precipitated with HOX11ΔC-terminus-HA but not with the HOX11L1/HOX11-HA chimaeric protein (Fig. 3d). GVBD was also stimulated by injection of RNA encoding the HOX11 N terminus (amino acids 1–190) (Fig. 3f). Therefore the homeo-domain of HOX11 was not required to stimulate GVBD. The effect of HOX11 on the endogenous phosphatase activity of injected frog oocytes was also assessed. HOX11 was approximately half as effective as okadaic acid in suppressing phosphatase activity for the substrate, phosphorylase *a* (Fig. 3g). These data are consistent with, although they do not directly prove that, HOX11 interaction with PP2A/PP1 results in the induction of GVBD.

To assess the effect of HOX11 on the mammalian cell cycle, the Jurkat T cell line was selected because after γ-irradiation it arrests at G2 (Fig. 4a). Jurkat cells were transiently transfected with a vector

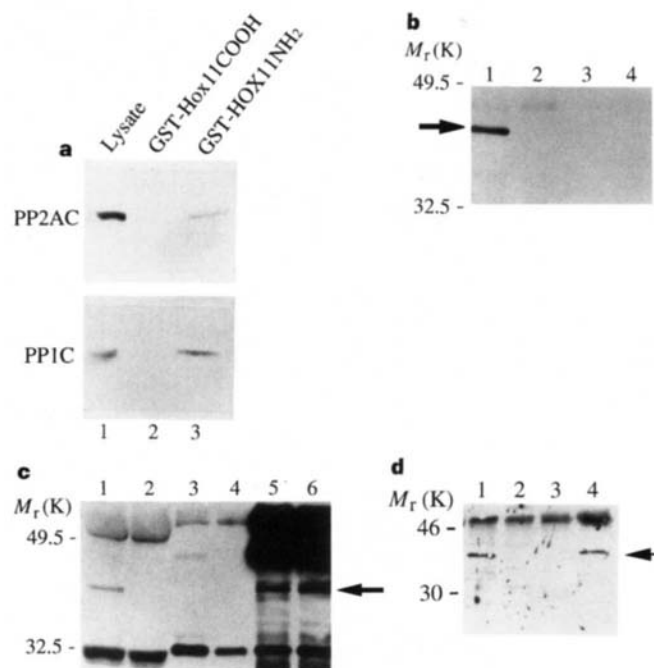


Figure 2 Association of HOX11 with PP2A and PP1 in mammalian cells. **a**, Western blot analysis of the PP2A and PP1 captured from Jurkat lysates by GST-HOX11 fusion proteins. Lanes 1, Jurkat lysate; 2, eluate from GST-HOX11 C-terminus (amino acids 261–330) column; 3, eluate from GST-HOX11 N-terminus column (amino acids 1–190). Jurkat lysates were incubated with GST-HOX11 fusion proteins at 27°C. **b**, ALL-Sil (lane 1) and Jurkat T leukaemia cells (lane 2) were immunoprecipitated with the 1D7 anti-HOX11 monoclonal antibody; a control anti-MLL monoclonal antibody was used in the primary immunoprecipitation of ALL-Sil (lane 3) and Jurkat (lane 4) cells. An immunoblot was developed with the 1D7 antibody. Arrow indicates HOX11 (Mr, 37K). **c**, Co-immunoprecipitation of PP2A with HOX11. ALL-Sil (lanes 1 and 3) and Jurkat (lanes 2 and 4) cells were immunoprecipitated with 1D7 monoclonal antibody (lanes 1 and 2) or anti-MLL monoclonal antibody (lanes 3 and 4). ALL-Sil (lane 5) or Jurkat (lane 6) cells were immunoprecipitated with anti-PP2AC antibody. Immunoblot was developed with the anti-PP2AC antibody. Arrow indicates PP2AC (Mr, 36K). **d**, Co-immunoprecipitation of PP1 with HOX11. ALL-Sil was immunoprecipitated with anti-HOX11 1D7 (lane 1), anti-PP2B (lane 2), anti-PP2AC (lane 3), and anti-PP1C (lane 4) antibodies. Immunoblot was developed with anti-PP1C antibody.

expressing HOX11ΔC-terminus-HA and given 5 Gy γ-irradiation. Cell-cycle analysis indicated that cells which do not express HOX11 (HA negative) accumulated at G2, whereas cells that expressed HOX11 (HA positive) do not arrest at G2 (Fig. 4b). A second approach used a LAP267-pL7 vector¹⁵ induced by isopropyl β-D-thiogalactopyranoside (IPTG) to express full-length HOX11. The Jurkat-Hox11-i subclone expressed low levels of HOX11 and responded to IPTG, but the Jurkat-n subclone did not express HOX11 before or after induction (Fig. 4c, d). After 5 Gy γ-irradiation, the Jurkat-Hox11-i clone had 15.8% cells in G1, whereas Jurkat-n had 3% (Fig. 4c). IPTG induction of HOX11 further increased the G1 population to 30% (Fig. 4d).

To determine whether HOX11-expressing cells that reside in the G0/G1 peak after γ-irradiation (Fig. 4) had actually violated a G2 checkpoint and traversed M phase, we labelled them with BrdU. Starting 24 h after γ-irradiation, a population of BrdU-positive cells with 2N DNA content accumulated in the induced Jurkat-Hox11-i line, and increased to 18% by 37 h (Fig. 5a–f). In contrast, far fewer BrdU-positive cells with 2N DNA content were noted after γ-irradiation of the Jurkat-n line, which does not express HOX11 (data not shown). This indicates that HOX11 expression can disrupt a G2 checkpoint and allow cells to proceed inappropriately through M phase.

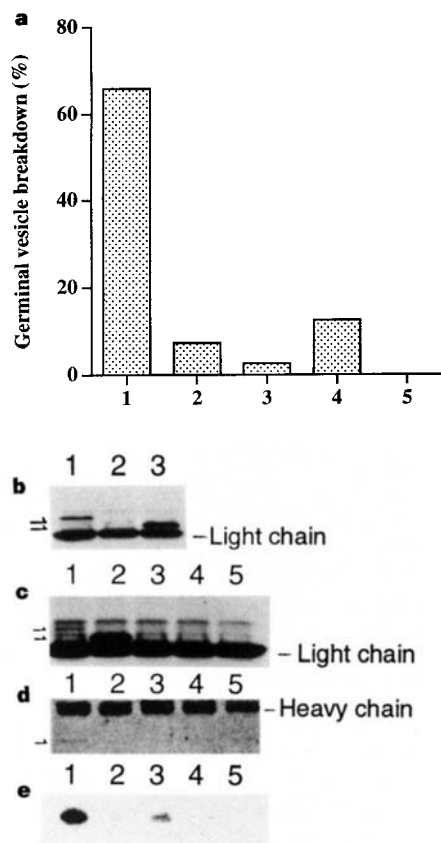
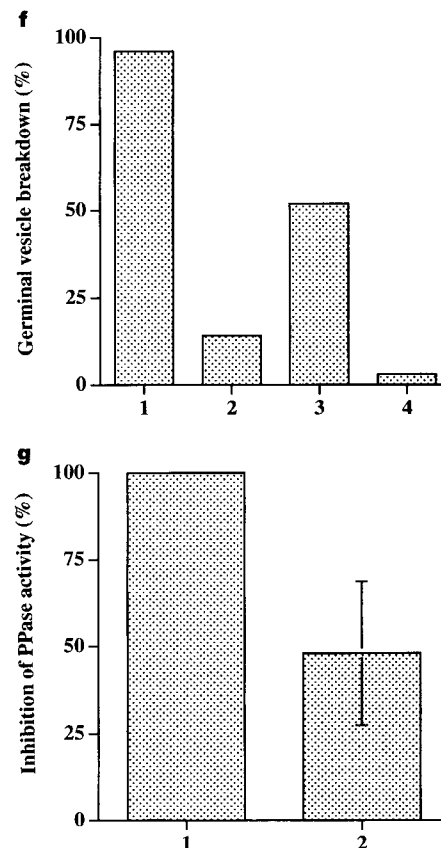


Figure 3 *HOX11* RNA microinjection into *Xenopus* oocytes. **a**, Percentage of germinal vesicle breakdown (GVBD) 24 h after microinjection of *HOX11*ΔC-terminus-HA (amino acids 1–263) sense RNA (column 1), *Hox11L1/HOX11*-HA sense RNA (column 2), antisense RNA (column 3), *HOX11* amino acids 1–124 sense RNA (column 4), and mock-injected control cells (column 5). A representative of multiple independent experiments is shown. **b**, Western blot analysis of *in vitro* translated *HOX11*ΔC-terminus-HA (amino acids 1–263) sense RNA (lane 1), antisense RNA (lane 2) and *Hox11L1/HOX11*-HA sense RNA (lane 3) immunoprecipitated and developed with anti-HA monoclonal antibody. Arrows indicate expected sized proteins. **c**, Western blot analysis of injected frog lysates. Lanes 1, *HOX11*ΔC-terminus-HA sense RNA; 2, *Hox11L1/HOX11*-HA sense RNA; 3, antisense RNA; 4, *HOX11* amino acids 1–124 sense RNA; 5, mock injection; immunoprecipitated and developed with anti-HA monoclonal antibody (12CA5). **d**, Co-immunoprecipitation of PP2AC with *HOX11*ΔC-terminus-HA. Lanes 1, *HOX11*ΔC-terminus-HA sense RNA; 2, *Hox11L1/HOX11*-HA sense RNA; 3, antisense RNA; 4, *HOX11* amino acids 1–124 sense RNA; 5, mock injection. Primary



immunoprecipitation used anti-HA monoclonal antibody (12CA5), and the immunoblot was developed with an anti-human PP2AC antibody that reacts weakly but consistently with *Xenopus* PP2AC. **e**, Histone H1 kinase assay of injected frog lysates. Lanes 1, *HOX11*ΔC-terminus-HA sense RNA; 2, *Hox11L1/HOX11*-HA sense RNA; 3, antisense RNA; 4, *HOX11* amino acids 1–124 RNA; 5, mock injection. **f**, Percentage of GVBD 24 h after microinjection of okadaic acid (column 1), mock injection control (column 2), *HOX11*-N-terminus (amino acids 1–211) sense RNA (column 3), and an empty vector (column 4). **g**, Percentage inhibition of the phosphatase activity in microinjected *Xenopus* oocyte lysates for phosphorylase *a* substrate. The microinjection of okadaic acid into frog oocytes inhibited 49% of the total phosphorylase *a* phosphatase activity present in lysates prepared 24 h after microinjection. For comparison, the percentage inhibition of phosphatase activity by the microinjection of okadaic acid was set at 100% (column 1). The comparative suppression of phosphatase activity by injected *HOX11* represents the average of three independent experiments (column 2).

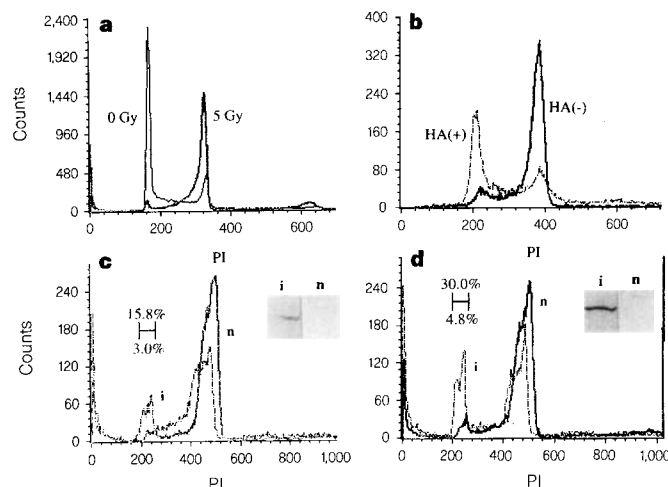


Figure 4 Expression of *HOX11* in Jurkat T cells. **a**, Propidium iodide (PI) staining of Jurkat cells before (0 Gy) and 20 h after 5 Gy γ -irradiation. **b**, PI pattern of Jurkat cells 20 h after 5 Gy γ -irradiation gated for HA-positive and HA negative populations after transient transfection of pRep9-*HOX11*ΔC-terminus-HA. **c**, A Jurkat HOX11-i subclone bearing an IPTG-inducible construct expressing full-length *HOX11*-HA tag, and subclone Jurkat-n, which was non-inducible, were assessed by PI staining 24 h after 5 Gy of γ -irradiation. **d**, The same experimental paradigm as in **c** except that both clones were incubated with 10 mM IPTG for 3 days before γ -irradiation. The immunoblot band shown in **c** and **d** is the detection of the HA tag on *HOX11* in Jurkat-Hox11-i or -n. The brackets in **c** and **d** represent the percentage of cells in the G0/G1 peak.

Transforming viruses often encode proteins that interact with PP2A or PP1, including SV40 small t (ref. 16), Polyoma small and middle T (ref. 16), and adenovirus E4orf4 (ref. 17) and Epstein Barr virus EBNA2 (ref. 18). Several of these proteins are indispensable for transformation, and SV40 small t has been demonstrated to inhibit PP2A activity¹⁹. The tumour promoters okadaic acid, dinophys-toxin-1 and calyculinA inhibit both PP2A and PP1 (ref. 8). INH, a form of PP2A in *Xenopus* oocytes, can prevent MPF activation *in vitro*^{20,21}. Consistent with this, okadaic acid promotes mitosis in G2-arrested BHK1 cells²², and ablation of PP2A in yeast results in a semi-*wee* phenotype²³.

Human CDC25 phosphatase has been shown to cooperate in cellular transformation²⁴. In this context, our results suggest that HOX11 inhibits PP2A and perhaps PP1, abrogating a G2 checkpoint that could promote genomic instability and oncogenesis. Homeobox proteins may normally promote proliferation, as well as pattern cell fate. Generation of new structures during embryogenesis requires substantial cell proliferation. *Hox11*-deficient mice lack a spleen¹, and double knockouts such as *hox a-3^{-/-}/d-3^{-/-}* mice lack the entire atlas vertebral segment²⁵. In turn, loss-of-function mutations of PP2A in *Drosophila* alter neural cell fate²⁶. Interaction of PP2A with the N terminus of HOX11 implies a direct mechanism that is independent of the DNA-binding homeodomain. Similarly, a transcriptionally crippled *fushi tarazu* mutant lacking a homeo-domain still exerts a phenotype in *Drosophila*²⁷. Our results support a model in which protein-protein interaction between a homeobox and PP2A can alter cell-cycle regulation. □

Methods

Yeast two-hybrid method. A mouse mature T-cell cDNA library in Gal4 activation domain vector pACT was screened by the yeast two-hybrid method

using the HOX11 N terminus (amino acids 1–211) fused with the Gal4 DNA-binding domain in the pAS1 vector²⁸. PP2A catalytic subunits α and β were both isolated in 10^4 colonies screened. Gal4 DNA-binding domain vectors were constructed by using either PC97, PC86 or AS1 fused with deletion mutants of *HOX11*. Yeast Y190 was co-transformed with pACT-PP2AC and *HOX11* vectors.

Precipitations and immunoblots. GST–HOX11 fusion protein (20 μ g) attached to a GSH-agarose column was mixed with Jurkat lysate (2×10^7 cells in 2 ml, 0.25% NP-40, PBS) for 2 h. Proteins were eluted with 50 mM GSH, 10 mM Tris, pH 8.0. Immunoblot was developed with a rabbit anti-PP2AC polyclonal antibody or anti-PP1C polyclonal antibody by ECL (Amersham). Cells (10^8) were lysed in Lysis 250 buffer and immunoprecipitated with the primary antibody, followed by SDS–PAGE and immunoblotting developed with the 1D7 monoclonal antibody, anti-PP2AC or anti-PP1C polyclonal antibody followed by ECL.

Oocyte microinjection and GVBD. Stage VI oocytes were removed from adult female *Xenopus* and treated with collagenase²⁹. *HOX11* Δ C-terminus without the acidic C-terminal domain with an haemagglutinin epitope (HA) was inserted into pSP64TS. *Hox11L1*/HOX11–HA was constructed by exchanging the N-terminal portion of HOX11 (amino acids 1–199) with HOX11L1 N terminus (amino acids 1–145)¹⁴. *In vitro* transcribed, capped RNA (1 μ g μ l⁻¹, 50 nl) or okadaic acid (2 μ M, 50 nl) was injected into *Xenopus* oocytes and 40 cells were scored for GVBD 24 h after each treatment.

PP2A plus PP1 phosphatase activity. For assessment of phosphatase activity, *Xenopus* oocytes were collected 24 h after microinjection and lysed with 137 mM NaCl, 0.5% NP-40, 10 mM Tris–Cl, pH 7.5, 2 mM phenylmethylsulphonyl fluoride, 0.2 U of aprotinin per ml, and 25 μ M leupeptin. Phosphorylase *a* served as the assay substrate (GibcoBRL) for the PP2A plus PP1 phosphatase activity in *Xenopus* lysates. The suppression of phosphatase activity in the okadaic acid-injected oocyte lysates as compared to antisense RNA-injected control oocyte lysates represented 100% inhibition. Histone H1 kinase assay was performed³⁰.

HOX11 expression vector in Jurkat cells. Jurkat cells were transfected with the expression vector pRep9 (Invitrogen) with HOX11 Δ C-terminus (amino acids 1–263) fused with the haemagglutinin epitope tag (HA). Cells received 5 Gy γ -irradiation 24 h after transfection, and were cultured for 20 h. Cells were then fixed with 70% ethanol, washed with 0.1% Tween 20–PBS, treated with RNaseA (Sigma), stained with 12CA5 monoclonal antibody specific for HA (Babco) plus FITC-labelled goat anti-mouse IgG (Tago) as well as PI, and analysed by FACScan (Beckton Dickinson).

Inducible HOX11 clone and BrdU. Jurkat *Hox11-i* was induced with 10 mM IPTG for 3 days and subjected to 5 Gy γ -irradiation; BrdU was immediately added at a final concentration of 20 μ M. Cells were fixed with 70% ethanol, treated with RNaseA at 37 °C for 15 min, denatured with 4 M HCl for 20 min, neutralized with 0.1 M Na₂B₄O₇, washed with 0.5% Tween20–PBS, and stained with an anti-BrdU monoclonal antibody conjugated with fluorescein isothiocyanate (Caltag) and 200 μ g ml⁻¹ PI. The cells that were stained only with PI were not subjected to the HCl treatment.

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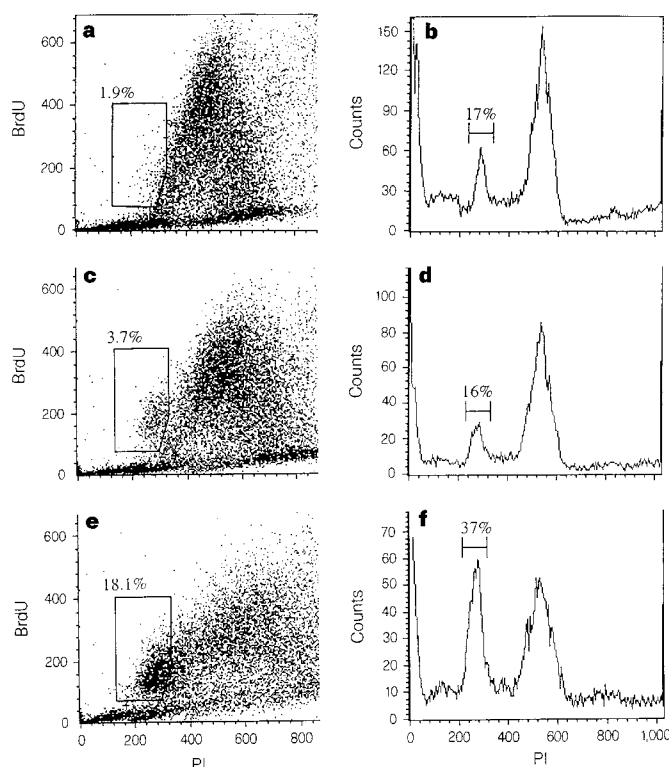


Figure 5 Induction of HOX11 in Jurkat violates G2 arrest. **a, c, e**, BrdU plus PI double staining of IPTG-induced Jurkat-Hox11-i analysed 19 h (**a**), 24 h (**c**) and 37 h (**e**) after 5 Gy γ -irradiation. The population with clear BrdU positivity and 2N DNA content is boxed. **b, d, f**, Jurkat-Hox11-i induced with IPTG was stained with PI alone 19 h (**b**), 24 h (**d**) and 37 h (**f**) after 5 Gy γ -irradiation. The presence of BrdU slowed the cycling of cells. Brackets indicate the percentage of cells in the G0/G1 peak.

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The structure of the GTPase-activating domain from p50rhoGAP

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Members of the Rho family of small G proteins transduce signals from plasma-membrane receptors and control cell adhesion, motility and shape by actin cytoskeleton formation^{1–4}. They also activate other kinase cascades. Like all other GTPases, Rho proteins act as molecular switches, with an active GTP-bound form and an inactive GDP-bound form⁵. The active conformation is promoted by guanine-nucleotide exchange factors, and the inactive state by GTPase-activating proteins (GAPs) which stimulate the intrinsic GTPase activity of small G proteins⁶. Rho-specific GAP domains are found in a wide variety of large, multi-functional proteins⁷. Here we report the crystal structure of an active

242-residue C-terminal fragment of human p50rhoGAP⁸. The structure is an unusual arrangement of nine α -helices, the core of which includes a four-helix bundle. Residues conserved across the rhoGAP family are largely confined to one face of this bundle, which may be an interaction site for target G proteins. In particular, we propose that Arg 85 and Asn 194 are involved in binding G proteins and enhancing GTPase activity.

In addition to their GAP activity domains, rhoGAP proteins can contain SH2, SH3, Ser/Thr kinase, and pleckstrin homology domains, as well as proline-rich regions^{7,8}. Several of these domains are known to mediate protein–protein interactions. With the exception of the chimerins⁹, rhoGAPs are ubiquitously expressed and so require tight regulation to prevent permanent deactivation of Rho-family GTPases. The coupling of protein–protein interaction domains to rhoGAP activity probably provides an indirect means of regulation through control of its subcellular location.

The 439-residue p50rhoGAP molecule is a relatively small member of the rhoGAP family. A 242-residue fragment of p50rhoGAP that contains 45 residues N-terminal to the rhoGAP domain⁸ was crystallized. The structure of this fragment has been solved by a combination of single isomorphous replacement, multi-wavelength anomalous scattering and two-crystal real-space averaging and has been refined at a resolution of 2.0 Å (Table 1). The structure consists of nine α -helices, labelled in Fig. 1a as A0 to G. The core of the structure is formed by the A–B and E–F α -hairpins; these four helices are arranged as a four-helix bundle. Helices A and B are separated by a 38-residue segment containing within it the A1 helix, which packs across the A and B helices. Extending from the core structure on the opposite side to helix A1 is a further α -hairpin made up of helices C and D. The E–F and C–D helical pairs are arranged as a cradle in which the final helix, G, sits roughly perpendicular to its packing mates. The domain is arranged such that the molecule possesses two large faces, one of which is dominated by the B and F helices (Fig. 2a). Packing of the B and F helices generates a shallow depression between them (Fig. 1b), the floor of which is made up of largely hydrophobic residues. A loop between the B and C helices defines the lower extent of this groove with the A–B and F–G loops closing it at the top. A similar structure has been determined¹⁰ for a domain in the p85 subunit of phos-

Table 1 Summary of crystallographic analysis

Table 1. Summary of crystallographic analysis									
Crystal	Native		CdCl ₂	Se-Met ($\lambda = 1.54$ Å)	Se-Met ($\lambda_2 = 0.9788$ Å)	Se-Met ($\lambda_3 = 0.97787$ Å)	Se-Met ($\lambda_4 = 0.9$ Å)		
Resolution (Å)	P2 ₁ 2.7	P2 ₁ 2 ₁ 2 ₁ 2.0	2.5	2.5	3.30	3.30	3.30		
Unique reflections	12,336	14,248	6,815	8,141	3,009	3,106	2,752		
Completeness (%)	88	99.7	91.8	100.0	91.7	94.8	84.3		
Redundancy	1.9	3.5	3.5	7.5	2.5	7.0	2.3		
R _{merge} (%) (outer shell)	0.06 (0.22)	0.097 (0.38)	0.036 (0.09)	0.082 (0.17)	0.065 (0.10)	0.072 (0.10)	0.056 (0.09)		
Phasing statistics (20–3.3 Å)									
Sites			4	2	2	2	2		
R _{cullis}			0.87	0.81	0.94	0.92	0.95		
F.o.m.	0.51								
			α	β	γ	t1 (Å)	t2 (Å)	t3 (Å)	Correlation coefficient
Averaging transformations	ortho → mono	Mol A	159.3	– 157.8	– 167.6	30.89	– 6.84	– 10.60	0.93
	ortho → mono	Mol B	174.2	– 157.3	– 172.6	12.57	– 31.06	18.12	0.92
Refinement statistics									
Resolution (Å)	Atoms	Solvent molecules	R _{cryst} (%)	R _{free} (%)	R.m.s. bond lengths (Å)		R.m.s. bond angles (deg)		
20–2.0	1,565	212	22.0	28.3	0.016		1.8		

$R_{\text{merge}} = \sum_i |I_i - \langle I \rangle| / \sum_i I_i$, where I_i is the observed intensity and $\langle I \rangle$ is the average intensity. $R_{\text{cryst}} = \sum_i |F_o - F_{\text{calc}}| / \sum_i F_o$ for all reflections.

R_{free} is the same as R_{cryst} but calculated on the 5% of data excluded from refinement.

$R_{\text{cullis}} = \sum_i |F_{\text{ph}} \pm F_p| - |F_{\text{hc}}| / \sum_i |F_{\text{ph}} \pm F_p|$, where F_{ph} and F_p are the derivative and native structure factors, and F_{hc} is the calculated heavy-atom structure factor.

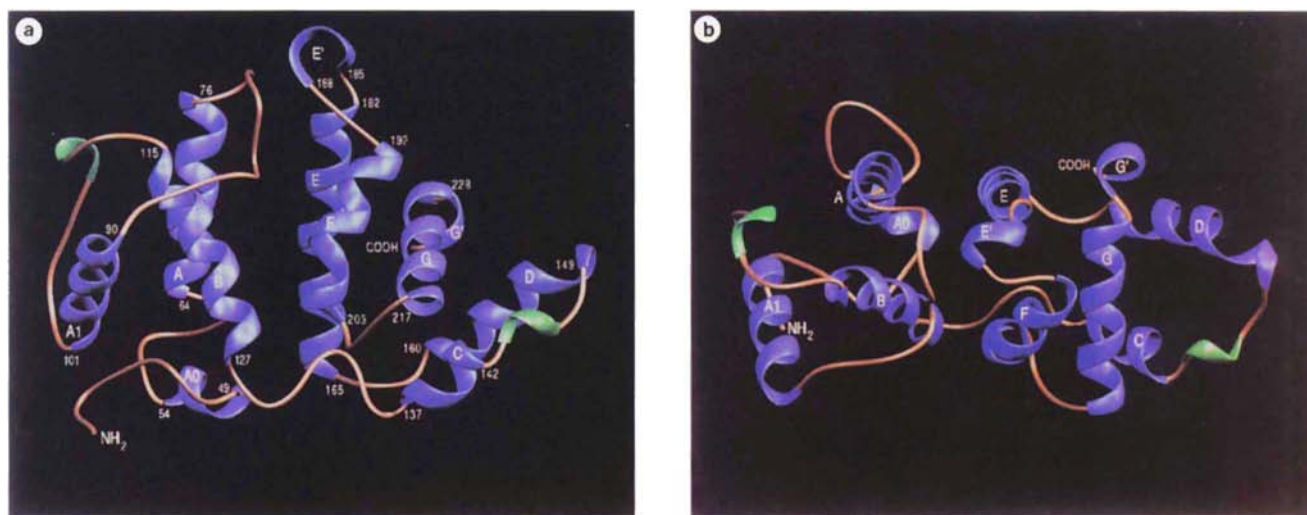


Figure 1 Ribbons¹⁹ representation of the GAP fragment of p50rhoGAP. **a**, The molecule is viewed from the side looking into the B-F face. The helices are labelled from N to C terminus as A0 to G. There are nine helical segments and two short helical turns (labelled E' and G'), all of which are shown with the starting and

ending residues numbered. Two short regions of 3_{10} helix between helices A1 and B, and helices C and D, are shown in green. **b**, The molecule is viewed after rotation of 90° about the horizontal, showing the four helical bundle (helices A, E, B and F) and the pocket-like depression on the B-F surface.

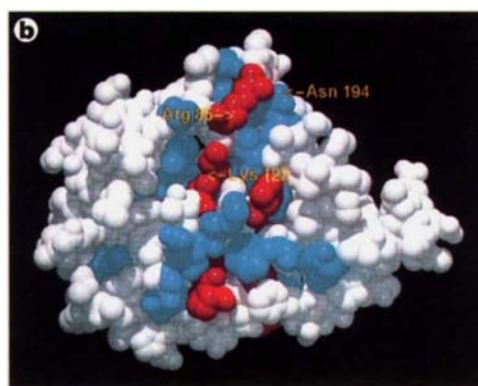
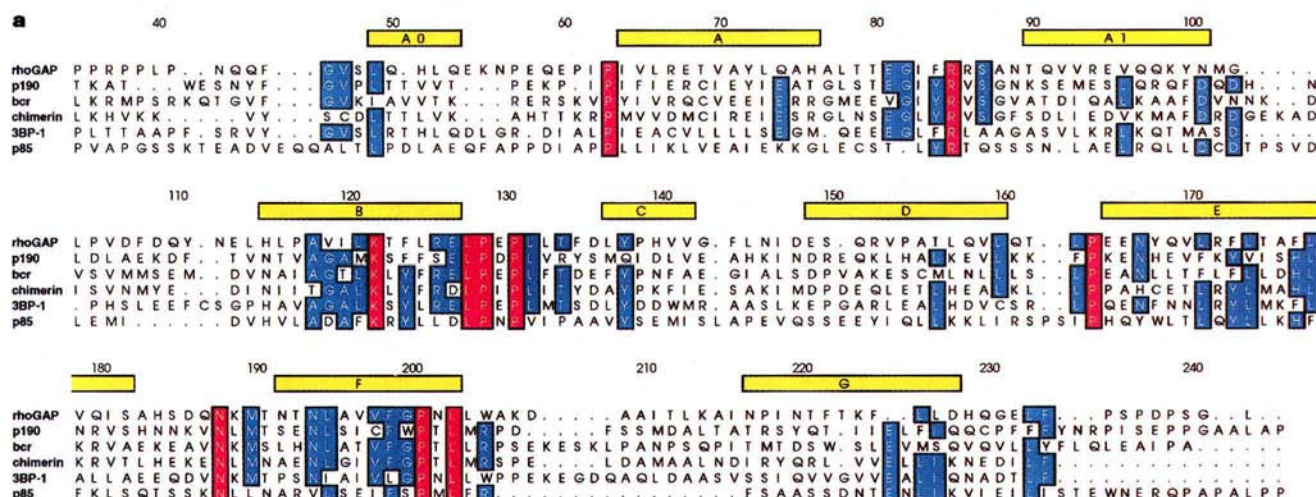


Figure 2 a, Sequence alignment of six members of the rhoGAP family as aligned using the Clustal algorithm and displayed using ALSCRIPT. The sequences are: p50rhoGAP (ref. 8) (Z23024), p190 (ref. 19) (A38218), Bcr (ref. 20) (A28765), n-chimerin (ref. 9) (P15882), 3BP-1 (ref. 21) (X87671) and p85 α (ref. 22) (P27986). Residues identical in all six family members are shown in red, with residues identical in at least four of the proteins shown in blue. The helical segments labelled in Fig.1 are shown in yellow. The numbering is such that the first residue

in the fragment used here corresponds to residue 198 in full-length p50rhoGAP. Residues N-terminal to 35 are not seen in the electron density, are not conserved, and are not included in the alignment. **b**, Space-filling model of the rhoGAP domain viewed in the same orientation as Fig.1a. In accordance with **a**, identical residues are shown in red with highly conserved residues in blue. Arg 85, Lys 122 and Asn 194 are labelled.

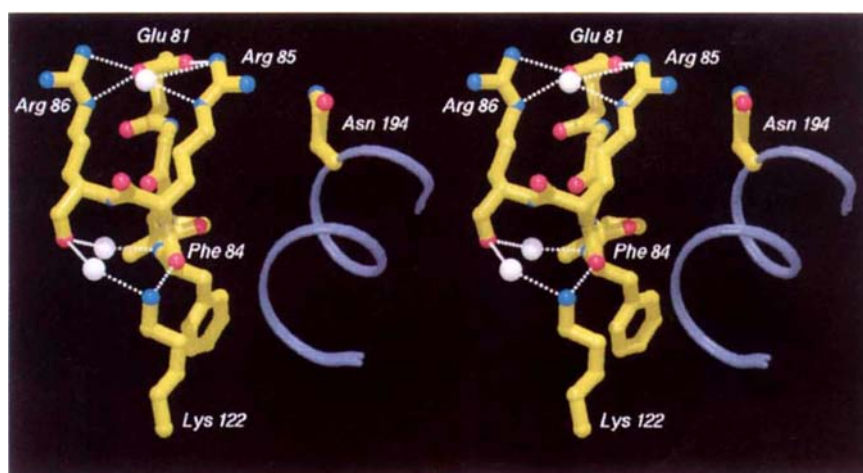


Figure 3 Stereo view of the structural interactions around the absolutely conserved residues Arg 85 and Lys 122. The backbone conformation of the A–A1 loop is stabilized in this region by hydrogen bonding interactions to Lys 122 and through hydrophobic interaction of Phe 84 with non-polar residues in the E

and F helices (not shown). Asn 194, which is absent only in the p85 protein (which has no GAP activity), is located at the C-terminal end of the E helix, in close proximity to Arg 85. The colouring convention is: carbon, yellow; nitrogen, blue; oxygen, red; water molecules are shown as white spheres.

phatidylinositol-3-OH kinase; it is homologous to the rhoGAP domain described here, but lacks GAP activity¹¹.

Analysis of sequence conservation amongst proteins containing rhoGAP domains suggests that the B–F helix pocket is important in G-protein binding. There are just 10 identical residues (shown in red in Fig. 2b) and 42 that are conserved (shown in blue). These two categories of residues are shown with the same colour coding in the space-filling model of Fig. 2a. The distribution of these conserved residues correlates strongly with the core of the domain in general, and the B–F pocket in particular. Of the ten totally conserved residues, seven are proline or leucine and are important for the structural integrity of the molecule. Indeed two of the core helices (B and F) each contain one of these conserved prolines. The remaining three conserved residues are Asn 188, Lys 122 and Arg 85. Asn 188 is located in the E–F loop and is important for its stabilization through main-chain hydrogen bonding with Ile 181 and Met 190. Lys 122 is located on the B-helix and is solvent exposed. It is involved in a hydrogen-bonding network with main-chain groups of Phe 84, Arg 85 and Arg 86 on the A–A1 loop (Fig. 3). Phe 84 is, itself, involved in extensive hydrophobic packing interactions with Phe 177 and Phe 199 on helices E and F, respectively, and anchors the A–A1 loop to the core. This array of interactions orders the A–A1 loop and consequently orientate Arg 85, whose side chain is involved only in bonding contacts to non-conserved residues. These data lead us to conclude that Arg 85 is very likely to be involved in G-protein binding to GAPs.

Of the six aligned sequences shown in Fig. 2b, all represent proteins that bind to one or more of the Rho family of G proteins, but only five of them enhance GTPase activity. The sixth protein, p85, does not. There are five residues conserved in the proteins that promote GTP hydrolysis that are not conserved in p85: Gly 82, Leu 132, Leu 178, Met 190 and Asn 194. The first four of these are located around the B–F pocket but are non-polar; Asn 194 is situated on the F helix with its side chain close to that of Arg 85. The chemistry and location of this residue suggests that it plays an important role in GAP function. Site-directed mutagenesis will now enable us to test whether rhoGAP participates directly in chemical catalysis or acts allosterically on intrinsic GTPase activity. □

Methods

Crystallization. The rhoGAP fragment (198–439) was expressed and purified⁸. Two crystal types were produced by vapour diffusion; monoclinic crystals were grown at 4 °C from 3% PEG 8K, 30 mM zinc acetate, 170 mM sodium cacodylate, pH 6.7, using a protein concentration of 4 mg ml⁻¹. Orthorhombic crystals were grown from 10% PEG 6K, 5% MPD in 0.1 M HEPES, pH 7.5, at

18 °C. The monoclinic crystals belong to space group *P*₂₁ with *a* = 60.7 Å, *b* = 64.4 Å, *c* = 67.7 Å, β = 99.4° and contain two molecules per asymmetric unit, whereas the orthorhombic form belongs to space group *P*₂₁₂₁ with *a* = 35.9 Å, *b* = 70.0 Å, *c* = 78.8 Å with one molecule per asymmetric unit. Crystals of the orthorhombic form were also grown from seleno-methionine substituted protein.

Data collection and processing. All diffraction data sets were measured from single crystals at 100K using an Oxford Cryosystems device. The crystals were prepared for flash cooling by immersion in mother liquor made up to 20% glycerol. A data set for the monoclinic crystals was collected on a RaxisII image plate mounted on a Rigaku RU200HB generator. Datasets for native, CdCl₂ soaked and Se-methionine crystals of the orthorhombic form were also collected in-house to provide MIR phases. Beamlines X11 and X31 at EMBL Outstation, DESY, were used to collect higher-resolution native and multi-wavelength datasets for the Se-methionine crystals. The optimal wavelengths for Se data collection at DESY were determined by measuring a fluorescence scan. The data were then collected at 0.97880 Å (λ ₂) and 0.97787 Å (λ ₃) to optimise *f'* and *f''*, respectively, and then at a remote wavelength 0.9 Å (λ ₄) for MAD phasing. Data were processed with DENZO¹² and SCALEPACK¹², and all subsequent calculations were carried out with the CCP4 program suite¹³.

Structure solution and refinement. Initial Cd²⁺ sites were found by using HASSP¹⁴, and further Cd²⁺ and Se sites were identified by difference Fourier analysis. Heavy-atom parameters were refined using MLPHARE¹⁵ and the resulting phases further refined using DM¹³. Seven of the eventual nine α -helices were built into the DM-derived map using O¹⁶. This partial model was used for molecular replacement on the monoclinic dataset using AMORE¹³. The two datasets were then real-space averaged using DMMULTI¹³ and the resulting phases, together with the preliminary model, were used with ARP¹⁷ to produce a greatly improved electron-density map. At this point all of the rhoGAP fragment was built into the map except for the first 34 N-terminal residues, including part of the poly-proline sequence which appears to be disordered, and 8 residues missing from the F–G loop. Subsequent model refinement was carried out using REFMAC¹³.

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Correspondence and requests for materials should be addressed to G.G.D. (e-mail: g-dodson@anika.nimr.mrc.ac.uk). Coordinates have been deposited in the Brookhaven Protein Data Bank, accession number 1RGF.

Crystal structure of colicin Ia

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The ion-channel forming colicins A, B, E1, Ia, Ib and N all kill bacterial cells selectively by co-opting bacterial active-transport pathways and forming voltage-gated ion conducting channels across the plasma membrane of the target bacterium^{1,2}. The crystal structure of colicin Ia reveals a molecule 210 Å long with three distinct functional domains arranged along a backbone of two extraordinarily long α -helices. A central domain at the bend of the hairpin-like structure mediates specific recognition and binding to an outer-membrane receptor³. A second domain mediates translocation across the outer membrane via the TonB transport pathway⁴; the TonB-box⁵ recognition element of colicin Ia is on one side of three 80 Å-long helices arranged as a helical sheet. A third domain is made up of 10 α -helices which form a voltage-activated and voltage-gated ion conducting channel across the plasma membrane of the target cell. The two 160 Å-long α -helices that link the receptor-binding domain to the other

domains enable the colicin Ia molecule to span the periplasmic space and contact both the outer and plasma membranes simultaneously during function^{6,7}.

The structure of colicin Ia, a 626-amino-acid protein (M_r 69K), was determined to 3.0 Å resolution by multiple isomorphous replacement using mercury derivatives of site-directed Ser $\rightarrow$ Cys mutants, synchrotron radiation and cryocrystallography (Table 1). Electron density obtained after density modification, unbiased by an atomic model, allowed unambiguous interpretation of the connectivity of the polypeptide from residues 23–624 (Fig. 1). Colicin Ia is $\sim$ 210 Å long (Fig. 2). From the structure, each function of colicin Ia previously localized by proteolysis¹⁰, or by analogy to results of deletion analysis⁸ and chimaeric constructs⁹ of colicins A and E1, can now be associated with a distinct structural region. The positions of many of the helices observed here overlap helical regions previously seen in the partial structure at 4 Å resolution¹⁰, which revealed aspects of the domain structure of colicin Ia but neither connections between elements of the structure nor sequence assignments. The monomer has a high axial ratio, consistent with the unusually large axial ratio of $\sim$ 16:1 deduced from sedimentation behaviour assuming a prolate ellipsoid molecular shape¹¹.

Binding to the outer membrane receptor, Cir (ref. 3), is encoded in a central domain first defined in channel-forming colicins by chemical digestion of colicin E1 (ref. 12). The structure shows that this domain comprises 104 residues (282–385) located at one extremity of the molecule (Fig. 3). Residues 295 to 329 form a highly amphipathic two-stranded β -hairpin, which folds around helix C1 to display a high density of charged amino acids near its terminal loop. Asp 311 and Arg 313 are alongside Glu 357, Asp 358, Glu 360, Asp 362, Lys 363 and Lys 364 on the tip of the claw-like, receptor-binding region.

The N-terminal domain believed to mediate translocation across the outer membrane is composed of three α -helices which form an unusual side-by-side antiparallel helical sheet. Two of the helices, T1 (residues 67–121) and T2 (residues 126–168), are $\sim$ 80 Å long, and the third includes the first 50 residues (176–225) of helix T3 which extends to the receptor-binding (R) domain (Fig. 3). The TonB box⁵, a pentapeptide conserved in colicins that use the TonB pathway, is required for translocation; mutations in the TonB box prevent colicin translocation but not binding to the outer-membrane receptor¹³. The TonB box of colicin Ia, Glu-Ile-Met-Ala-Val (residues 23–27), is located at the end of an extended N-terminal peptide which interacts along one surface of the helical sheet (Fig. 2). The surface of the N-terminal domain opposite the TonB box is

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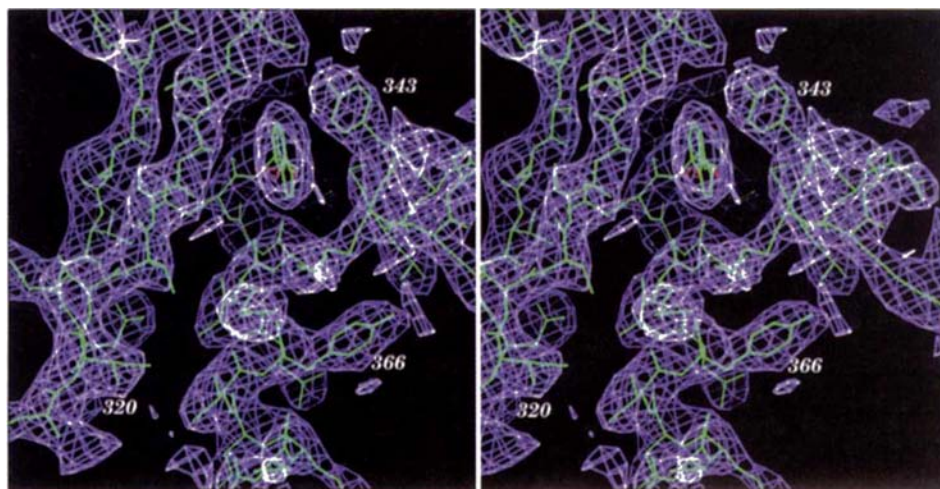


Figure 1 Stereoscopic image (divergent) of the SIGMAA²⁸-weighted $2F_o - F_c$ electron-density map contoured at 1.25σ at 3.0 Å resolution, with the current refined model depicted. Hydrophobic residues at the core of the R domain are

shown; Trp 373 on helix C1 is at the centre and the β -hairpin that stretches 45 Å across the domain is on the left.

highly charged, so that helices T1 and T2 have 15 intra- and inter-helical salt bridges along their length on that side.

The channel-forming domain comprises the C-terminal 176 residues (450–626) (Fig. 3). Two helices, C8 (580–594) and C9 (597–612), form a very hydrophobic hairpin sequestered in the centre of the domain. They are surrounded by eight amphipathic helices, C1 to C7 and C10, packed with their hydrophobic surfaces in contact with C8, C9, and giving the C domain a polar exterior. There is little, if any, sequence identity between different colicins; however, C1–C10 have the same general topology of helices as the channel-forming domain of colicin A (ref. 14). Upon initial electrostatic association with membranes, fluorescence labelling of the channel fragment of colicin A and resonance energy transfer spectroscopy show that, whereas helices C1 and C2 open out onto the membrane surface, the hydrophobic hairpin C8, C9 remains embedded in the helical bundle¹⁵. In a slow step, C8 and C9 of colicin E1 spontaneously insert into and span the bilayer as a hairpin¹⁶. The composition of C8 and C9 is consistent with that of transmembrane segments of integral membrane proteins, and C8, C9 on their own are sufficiently hydrophobic as to be insoluble even in organic solvents. The 34 amino acids that make up C8 and C9 are uncharged; 28 have hydrophobic side chains, including 7 leucines, 7 alanines, 4 isoleucines, 3 valines and 5 glycines.

Receptors that colicins parasitize are typically active-transport receptors that act as gated porins¹⁷. Binding of the natural ligand and subsequent transport are associated with a flap that lies above the pore¹⁸. After translocation of colicin Ia across the outer membrane, the channel domain acts on the plasma membrane. Spanning

the periplasmic space is facilitated by TonB⁴, a host integral plasma-membrane protein that normally couples the electrochemical gradient across the plasma membrane to active transport by outer-membrane receptors (Fig. 4). The conserved TonB box⁵ pentapeptide of both the channel-forming colicin and its receptor are essential for colicin uptake, suggesting that a competitive displacement of the receptor–TonB interaction by the TonB box of colicin Ia is required. After attack *in vivo*, the C-terminal domain associates with the plasma membrane initially by electrostatic association². Several of the amphipathic helices, C1–C7 and C10, open out onto the bi-layer^{15,19}. Subsequently, as determined by site-specific biotinylation^{20,21}, residues 474–541, now seen to be helices C2–C5, are spontaneously translocated across the bilayer in the presence of an applied transmembrane voltage. The voltage-dependent open-channel state is formed as electrostatic potential across the membrane drives a key portion of the 29 residues 544–572, recognized now as C6–C7, into the membrane²¹.

Channel-forming colicins contact the inner and outer membranes simultaneously during function^{6,7} and there is some contention that colicins require contact sites between the membranes to span the periplasmic space. In the structure of colicin Ia, the channel-forming and translocation domains are separated from the receptor-binding domain by ~160 Å long helices that form a hairpin, with the receptor-binding domain at the bend. The periplasmic space has an average width of ~150 Å (ref. 22). The 160 Å-long C1 (residues 359–467) and T3 (residues 176–282) helices present a clear mechanism for colicin Ia to span the periplasm while remaining in contact with both the inner and outer membranes.

The channels formed by a truncated C domain of colicin Ia alone differ in conductance from those of whole colicin Ia²³. Proteolytic protection in presence of lipid vesicles demonstrates that residues 358–626 and 79–190 are protected by association with lipids²⁴ (Fig. 3). However, several sites within this entire region remain accessible

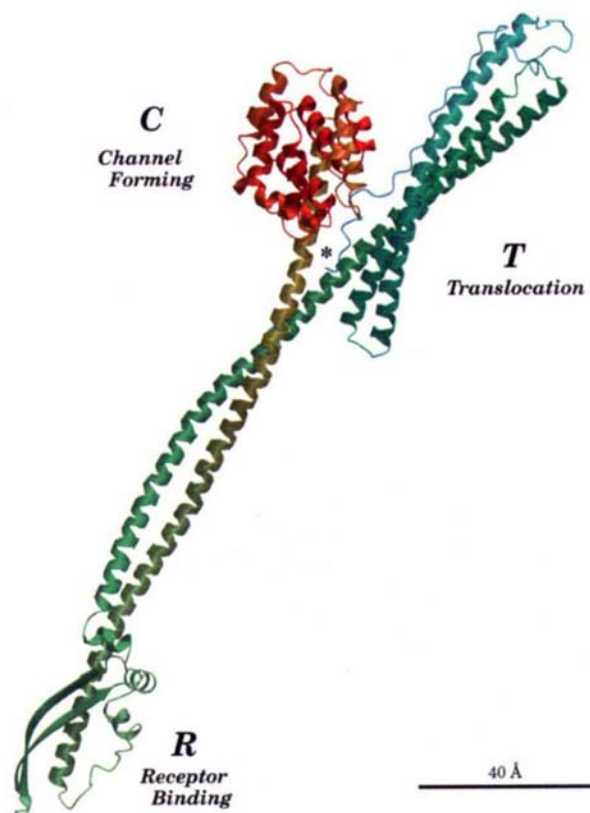


Figure 2 Ribbon representation of the structure of colicin Ia with blue-to-red colouring in the N-to-C direction. Scale bar, 40 Å. The molecule is ~210 Å long and consists of three functional domains separated by a pair of helices, each 160 Å long. The translocation (T), receptor-binding (R), and channel-forming (C) domains are indicated. Helices are numbered T1 to T3, R1 to R3, and C1 to C10 according to their location from N- to C-terminal. The location of the N-terminal peptide which includes the TonB box is marked by an asterisk between the T and C domains.

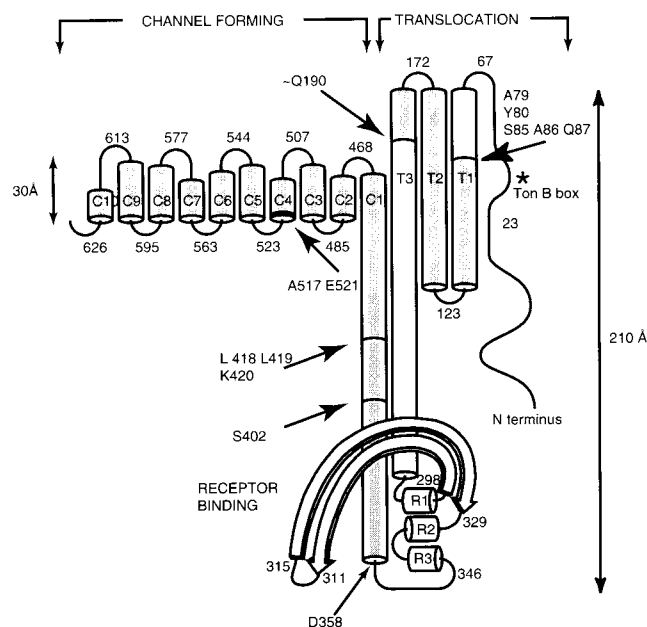


Figure 3 Topology of colicin Ia. The lengths of helices are to scale, with dimensions 210 Å indicated on the right, and 30 Å on the left. Association of colicin Ia with lipid bilayers *in vitro* leads to protection of several regions from proteases²⁴, indicated by shading along helices. Protected regions include residues 87–189, comprising most of the amphipathic helices T1, T2, and a portion of T3. This suggests that the amphipathic helices of the translocation domain may associate with the outer membrane after receptor binding to position and insert at least the TonB box through the receptor pore for the next step in translocation.

to proteolysis. These are Ser 402 and Leu418-Leu419-Lys420 in the long spanning helix C1, and Ala 517 to Glu 521, which lies at the end of C4. The majority of the long C1 helix and helices T1, T2 and T3 remain candidates for participating in channel formation *in vivo*. Although the long T3 helix has no obvious function, it could allow the translocation domain (T) to reach the inner membrane. □

Methods

The space group of the colicin Ia crystals is $C22_1$ ($a = 64.4 \text{ \AA}$, $b = 178.6 \text{ \AA}$, $c = 285.5 \text{ \AA}$). There is one monomer in the asymmetric unit, and a very high

solvent content of 78%. Crystals used for data collection were $\sim 0.8 \times 0.4 \times 0.01 \text{ mm}$. For the native dataset, freshly grown crystals were transferred to a harvest buffer of 1.1 M Na_2SO_4 , 200 mM NaCl, 20 mM sodium citrate, pH 5.2, 5 mM DTT. For derivatization, ten single-site cysteine mutants were constructed, expressed and purified as described¹⁰. Crystals of cysteine mutants were grown by cross-seeding. Drops of cysteine-mutant protein were equilibrated by hanging-drop vapour diffusion against reservoirs of 1.0 M $\text{NH}_4(\text{SO}_4)_2$, 20 mM sodium citrate pH 5.2, 200 mM NaCl starting from 6 μl protein at 2 mg ml⁻¹ in 20 mM Na-citrate, pH 5.2, 200 mM NaCl, 5 mM DTT and 4 μl reservoir solution. After 24 h equilibration, drops were streak-seeded

Table 1 Crystallographic statistics

Diffraction data												
Dataset	Resolution (Å)		Redundancy		Completeness (%)		R_{sym}^* (%)		$R_{\text{dt}}^\dagger$ (%)		$R_c^\ddagger$ (%)	
S171C (native)	3.0		3.4		84 (73)		13.0 (30.9)		-		-	
S171C + Hg	3.0		3.0		59 (51)		12.2 (29.1)		19.4		69	
S615C + Hg	3.0		3.3		74 (66)		13.4 (30.3)		17.1		69	
Phasing statistics												
	9.7	5.9	5.0	4.4	4.1	3.8	3.6	3.4	3.2	3.1	Total	
MIR:												
S171C + Hg	f_h/e	1.58	1.65	1.30	1.01	0.91	0.98	1.07	0.99	0.97	0.96	114
S615C + Hg	f_h/e	1.67	1.61	1.34	1.13	1.08	1.08	1.06	1.05	0.96	0.95	119
Figure of merit		0.41	0.30	0.35	0.36	0.34	0.30	0.27	0.24	0.22	0.19	0.30
After density modification:												
Phase shift		51.9	63.5	67.0	68.3	66.6	68.1	66.9	65.4	64.2	60.0	64.2
Figure of merit		0.82	0.86	0.88	0.88	0.86	0.83	0.81	0.78	0.77	0.71	0.82
Refinement statistics												
	$R_{\text{cryst}}^{\S}$ (%)	$R_{\text{free}}^{\parallel}$ (%)	Resolution (Å)		No. of reflections		No. of atoms	Bond length dev. (r.m.s.)		Bond angle dev. (r.m.s.)		
$F > \sigma F$	22.7 (24.1)	30.0 (31.2)	8.0 – 3.0		21,570 (26,689)		4,714	0.009 Å		1.48°		

Values in the high-resolution bin are shown in parentheses (diffraction data); refinement statistics, values for $F > 0$ are in parentheses.

$$*R_{\text{sym}} = \frac{\sum_i \sum_j |I_i(hkl) - I_j(hkl)|}{\sum_i \sum_j I_i(hkl)}$$

$$\dagger R_{\text{der}} = \frac{\sum_i |F_{\text{obs}}(hkl) - F_{\text{calc}}(hkl)|}{\sum_i |F_{\text{obs}}(hkl)|}$$

$$\ddagger R_c = \frac{\sum_i |F_{\text{obs}}(hkl) - F_{\text{calc}}(hkl)|}{\sum_i |F_{\text{obs}}(hkl)|} \text{ for centric reflections.}$$

$$\S R_{\text{cryst}} = \frac{\sum_i |F_{\text{obs}}(hkl) - F_{\text{calc}}(hkl)|}{\sum_i |F_{\text{obs}}(hkl)|}$$

$$\parallel R_{\text{free}} = \frac{\sum_i |F_{\text{obs}}(hkl) - F_{\text{calc}}(hkl)|}{\sum_i |F_{\text{obs}}(hkl)|} \text{ calculated with a 2,000-reflection test set.}$$

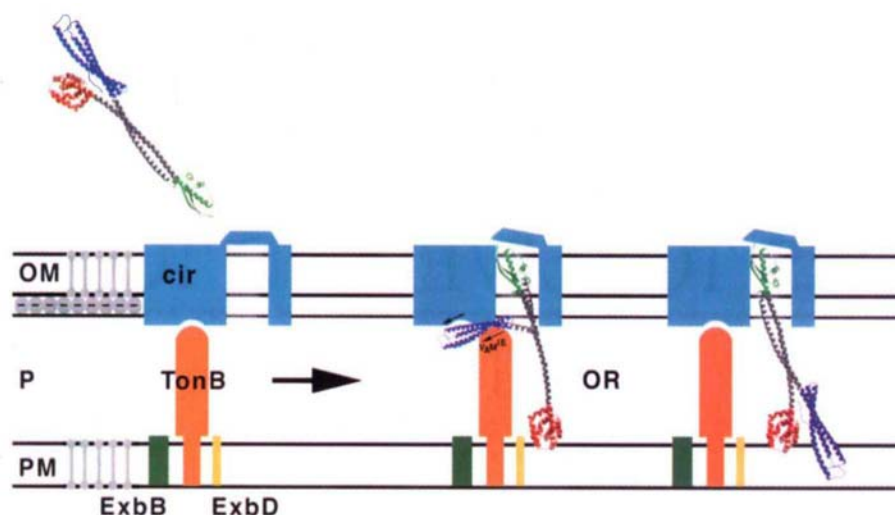


Figure 4 The mechanism of colicin Ia attachment and translocation; scheme is to scale. The outer membrane (OM) receptor for colicin Ia, Cir, and TonB and accessory plasma membrane (PM) proteins ExbB and ExbD are indicated. The TonB box of colicin Ia may compete with the TonB box of Cir for linkage to TonB. The channel-forming C domain reaches the plasma membrane where it forms an ion-conducting channel by subsequent insertion and rearrangement of helices within the membrane (not shown). The translocation T domain may remain near

the periplasmic surface of the outer membrane during channel activity. However, the presence of the 160 Å-long T3 helix indicates an alternative possibility in which the T domain crosses the periplasmic space (P) to participate in channel formation *in vivo*. The location of the TonB box on the upper surface of the T domain, of sequence Glu(E)-Ile(I)-Met(M) Ala(A)-Val(V) is indicated, reading right to left, as EIMAV. The arrows schematize the locations of the TonB box in the receptor, and colicin Ia.

from stocks generated from crushed diffraction-quality wild-type colicin Ia crystals¹⁰. The crystals were washed in DTT-free harvest buffer and stored for 1 week in DTT-free harvest buffer containing 1 mM CH₃HgCl. Two mutants, S171C and S615C, yielded useful derivatives.

Diffraction data. Data were collected at beamline 7-1 ($\lambda = 1.08 \text{ \AA}$) at the Stanford Synchrotron Radiation Laboratory (SSRL) on a 300-mm MAR Research image plate. Crystals were transferred to a cryosolvent of 70% harvest buffer and 30% glycerol, equilibrated for 5 min, and frozen in rayon loops in a 90 K N₂ coldstream. Data were reduced with DENZO/SCALEPACK²⁵.

MIR phasing. The Harker sections of the Hg difference-Patterson maps indicated single-site solutions, and the heavy-atom parameters (including anisotropic temperature factors) were refined with PHASES. The occupancies of both derivatives were similar. Subsequent map calculations were made using the CCP4 package. The figure of merit weighted MIR map allowed distinction between the protein and solvent regions of the crystal, but was otherwise not interpretable.

Density modification. Because of the very high solvent content, we expected that application of a solvent constraint could yield substantial improvement in the map. At 6 Å resolution, the SOLOMON²⁶ solvent 'flipping' protocol revealed tubes of electron density (characteristic of α -helices at the resolution) almost 150 Å long. The cluster of helices identified¹⁰ as the pore-forming 'C' domain was also clear in this map. We found it necessary to implement a resolution extension solvent modification protocol to obtain significant improvement in the 3.0 Å resolution map. The solvent fraction used was 70%, the solvent region flipping factor was approximately -0.75 (as set by the program), and the lowest 20% and highest 2% of the protein region density were truncated. Missing structure factors were not reconstructed. Fifty cycles at

6 Å resolution were followed by 300 cycles of density modification as the resolution was extended from 6.0 to 3.0 Å in 0.01 Å steps. At each step the solvent mask was recalculated using a radius equal to the resolution of the map. Structure factors calculated from the modified map were used to estimate the phases in the next resolution shell; these were combined with the MIR phase probabilities at that resolution. After 350 total cycles of solvent 'flipping', 50 cycles of solvent flattening led to a 3.0 Å modified map, which was interpretable.

Model building and refinement. The polypeptide chain was built into the density-modified map using program O (ref. 27). Identification of the amino-acid sequence was facilitated by the long α -helices, which allowed side-chain estimates to be made over very long stretches of the sequence. Almost 80% of the polypeptide could be built into the initial maps. Subsequently, phases calculated from partial models were combined with the MIR phase set to yield SIGMAA²⁸-weighted electron density maps which were then also subjected to density modification using SOLOMON. Several cycles of phase combination, density modification, rebuilding and refinement yielded the current atomic model. Crystallographic positional and slow-cooling refinement was carried out using X-PLOR²⁹. An anisotropic B-factor correction was applied to the diffraction data. Inclusion of individual atomic temperature factors during the final stages of refinement was validated by a significant decrease in the value of R_{free} . The average refined B-factor is 66 Å². The current model includes residues 23–624 (4,714 non-hydrogen protein atoms) which is 96% of the 626-residue (69K) protein. The program PROCHECK³⁰ was used to check the quality of the structure. Overall, the stereochemical parameters are better than expected for a model at this resolution; five residues in disallowed regions of the Ramachandran plot are located in poorly defined loop regions.

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Correspondence and requests for materials should be addressed to R.M.S. (e-mail: stroud@msg.ucsf.edu). Coordinates have been deposited in the Protein Data Bank and will be held for one year from publication.

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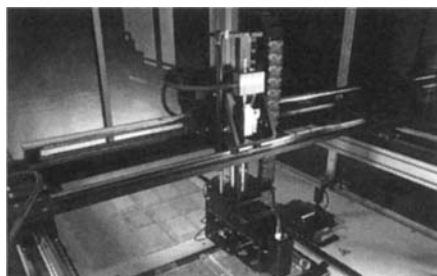
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Biotechnology in brief

Smaller, more versatile instruments and machinery continue to enter the market, including a high-throughput electronic dispenser, a precision capillary electrophoresis system and an FT-MS for analysis of biomolecules.



Mantis — Linear Drives' robotic processing

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plates at around 3,000 colonies per hour. With a hit rate of better than 98 per cent, over 9,000 colonies can be picked without user intervention. The system features automatic calibration of all vision and robotic parameters, as well as each individual pin on every picking head. It also has the ability to store and retrieve chosen picking parameters, coping with sloping or badly poured agar plates.

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MicroStar microbial detection

From Millipore

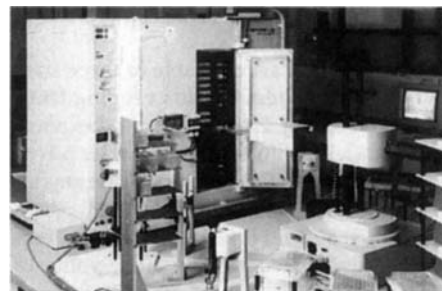
A rapid micro-detection system (RMDS) for QA-QC laboratories

This system is stated to provide yeast counts in as few as seven minutes and bacterial results in one-third the time of conventional tests. The new system offers microbiologists fast, reliable answers for the analysis of final product, raw materials, makeup water and rinse water. This product is designed to reduce the time spent on yeast and bacteria testing and, as a result, isolate contamination problems sooner. This product combines membranes with a bioluminescence assay and computer imaging software and hardware. The system incorporates membrane filtration to concentrate and separate the organisms from the sample for testing. It uses a Durapore membrane with more than 600 compartments separated by hydrophobic partitions. When using the luciferin-luciferase bioluminescence assay, ATP is used to determine the presence of organisms — the membrane is tested immediately after filtering the sample with yeast or after briefly incubating in the case of bacteria. After the assay, the RMDS system images the bioluminescence using an image intensification process, a charge couple device (CCD) camera and image analysis software. Computerized data handling improves analysis, image archiving and the tracking of trends; data can then be transferred to an electronic spreadsheet.

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Millipore's MicroStar microbial detection



Hereaus robotic, gassed incubation system

Cytomat 6000

From Heraeus Instruments

A robotic, gassed incubator for automated high-throughput random screening

This incubator has been engineered for automated high-throughput random screening (HTS) and uses a quick-reaction electromechanical conveyance system, which serves to accelerate the loading and unloading process. Now the microtitre plates are merely inserted and extracted through a narrow slit in the incubator. This process, together with the heating elements, helps to ensure that climatic parameters remain constant even at high-throughput rates. In this way, even sensitive assays can be processed. The core of this new apparatus is a coordinated transport and lift system that accepts the microtitre plates from the system robot and places them on and removes them from the microtitre plate carousel. The carousel-type magazine can accommodate up to 200 microtitre plates. Even with rapid loading sequences, the temperature of the work space is said to remain constant, which better simulates *in vivo* assay conditions.

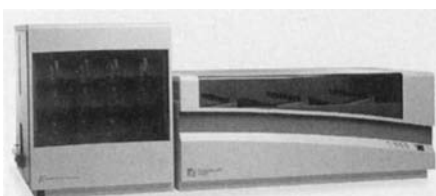
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Nautilus 2400

From Argonaut Technology

Automated organic synthesizer for lead optimization, chemistry development and resynthesis of compounds

This organic synthesizer is a modular, computer-controlled benchtop synthesizer with separate glass reaction vessels that operate under an inert, closed environment. It automates synthesis using non-robotic technologies that allow for continuous, unattended operation. The system offers flexibility with 24 independent glass reaction vessels whose temperatures can be individually regulated from -40 to 150 °C using a precise digital control system.



Argonaut's Nautilus 2400 organic synthesizer

Reaction vessels are available in three sizes allowing for product yields ranging from 30 mg to 350 mg. Fluid delivery takes place in a closed, inert environment under nitrogen or argon gas. Reagents and solvents can be delivered from up to 176 different reservoirs; flow of fluid is regulated by a proprietary valving technology and volumes ranging from 50 μ l to 5.8 ml are measured using the integrated sensor system. The system is computer controlled with all instrument control information contained in a Microsoft Access database, which allows users to enhance and modify many aspects of the software to their own specifications, as well as interface with other databases. The company states that the software follows the steps that a chemist would use to synthesize new compounds manually. A complete system consists of six principal components: the reaction vessel enclosure, reagent solvent enclosure, an autosampler, a fraction collector, a gas chiller and a computer. The instrument system fits into a 2.4-m fume hood or can be placed on a benchtop under a canopy hood.

Reader Enquiry No. 103

HP ChemLMS

From Hewlett-Packard Europe

SAP certification for its HP ChemLMS laboratory information management system (LIMS) interface

Based on the SAP QM-IDI interface, this system is stated to interface to QM, providing a seamless transfer of laboratory testing requests from QM to LIMS and a seamless return of valid laboratory test results to QM. This software integrates laboratory information into the enterprise, improving productivity, responsiveness and decision-making. According to the manufacturer, SAP certification ensures that users receive ready-to-use functional interfaces that will operate correctly with SAP installations by means of a thorough certification programme. Plug-and-play certified interfaces can shorten implementation times, reduce customers' costs and safeguard customers' investments in complementary products such as LIMS. This program is based on open-systems standards, such as the HP-OX(I) operating system, TCP/IP networking, Microsoft Windows 95/NT and the Oracle7 relational database. The ChemLMS interface to R/3 QM extends the system to include interaction with process

and quality management functions supported by SAP. The R/3 process modules automatically can trigger laboratory testing by creating inspection-lot requests through the QM interface to ChemLMS.

Reader Enquiry No. 104

EDOS 5222

From Eppendorf

A programmable, electronic dispenser for high-throughput manual dispensing tasks

Designed for high-throughput dispensing tasks, this system pipettes, dispenses, dilutes, titrates and mixes. It uses Combitips pipetting or multichannel adapters, which can be attached to the ergonomic dispensing grip. EDOS recognizes which adapter has been inserted to ensure the correct setting. Automatic Combitip ejection helps guarantee safety but also allows Combitips Plus to be used in a fully automatic operating system. The unit can also be controlled by a computer with the aid of the integrated interface; there is clear user prompting and useful direct-selection keys for all functions. Up to 30 work protocols can be stored for each mode and volumes between 0.5 ml and 50 ml can be selected. Users also have the option to calibrate for liquids with different viscosities.

Reader Enquiry No. 105



Eppendorf's EDOS 5222 liquid dispenser

Adeno-Quest transfer vectors

From Quantum Biotechnologies

Autofluorescent protein co-expression transfer vectors for rapid recombinant isolation using adenovirus expression systems

Designed for rapid recombinant isolation using an adenovirus expression system, these transfer vectors provide positive selection for the human adenovirus expression system. According to the manufacturer, the visualization capabilities using fluorescent, positive plaques could save the user up to one month on recombinant adenovirus purification. The proteins should also allow viral particle titration within 24 hours. Co-expression of the autofluorescent protein with the desired recombinant protein lets the user visualize the efficiency of transfection experiments both *in vivo* and *in vitro*.

Reader Enquiry No. 106

Tricentric Bioreactors

From Setec

High-performance, hollow-fibre bioreactors that can be used repeatedly

These bioreactors are designed for mammalian or insect cell culture for the production of either secreted products, such as monoclonal antibodies, or cells for harvest. They feature a fibre-within-fibre configuration to keep all cells immobilized and ensure shear-free conditions within 200 μ m of diffusible oxygen and nutrients. Applications include serum or serum-free culture, monoclonal antibodies, recombinant proteins, adherent and non-adherent cells, virus production, hybridoma, primary, CHO and insect cells. The bioreactors are available in 4-ml and 73-ml, as well as special sizes for use with NMR — all can be autoclaved, cleaned and reused.

Reader Enquiry No. 107

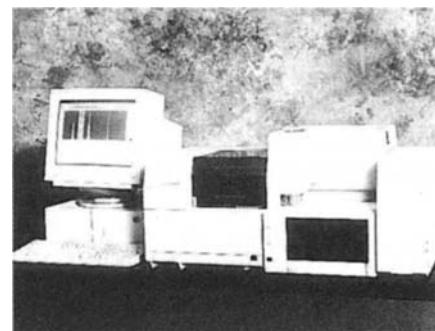
Capillary electrophoresis

SpectraPhoresis Ultra CE

From Thermo Separation Products

An automated, modular, precision capillary electrophoresis (CE) system

The system consists of a VialServer, the CE module and the W3000 scanning detector, all controlled by the company's PC1000 software. System precision is stated to be typically better than two per cent RSD of peak areas. Precision is achieved using temperature control around the capillary, control of the electrical resistance within the capillary, use of a special pressure injection process and by using a dual ball-lens that directs maximum light to the detector. With up to 110 vial positions, the system's VialServer offers sample thermostating, automatic buffer mixing, buffer replenishment and pre-capillary sample derivatization. Pre-capillary derivatization procedures are thoroughly automated to manage reagent addition, heating, cooling and vortex mixing. Bar-coded capillary cassettes track capillary histories, simplify record keeping and assist with documentation for good laboratory practices. The manufacturer states that the system accommodates any combination of chemistry, voltage, current, pressure and temperature for a wide



SpectroPhoresis Ultra CE modular system

range of applications. Moreover, the system will apply voltage or current at either end of the capillary. The software can be upgraded to provide simultaneous LC and CE operation. Automatic, interactive system-suitability calculations are tailored specifically to CE-LC. Although the system features the high-sensitivity UV 3000 scanning detector, fibre-optic sensing from the CE module facilitates the use of other detector methodologies, including CE-MS

Reader Enquiry No. 108

P/ACE Station software

From Beckman Instruments

A new Windows-based software package for the P/ACE Station capillary electrophoresis system

Designed to handle aspects of CE from data collection through to analysis and reporting, this system features calculations for apparent and electrophoretic mobility, corrected area, corrected area percent and corrected migration time. The software also calculates molecular weight and pI determinations. Other features include 'true-to-life' graphical instrument control for ease of use and minimal operator training. Existing P/ACE data files can be easily imported while post-run data functions evaluate up to 32 overlaid separations. Diode array data can be evaluated in a variety of ways: with three-dimensional plots, with simultaneous evaluation of individual wavelength data, with a two-dimensional contour plot or with individual absorbance profile on a single screen. Spectral libraries can also be stored for future search and reference. In addition, the software can export and import data to and from other applications and produces reports with customized features, including page layout and data fields. For compliance with good laboratory practices, the system includes all CE calculations, with three different levels of operational security.

Reader Enquiry No. 109

SIL-FC column and buffer system

From J&W Scientific

New buffer kits for pH stability from 2.5–10 in capillary electrophoresis

These kits contain columns, buffers and additives that work together, allowing the user to choose both pH and electroosmotic flow (EOF) to optimize separations. The column is the latest advance in coated capillary technology giving an effective pH stability from 2.5–10. Available buffer systems include phosphate, citrate and acetate — all of which feature an ease-to-use instruction manual and complete pH table for calculating pH for the selected buffer system. The company offers a 32-page application booklet illustrating applications of these kits.

Reader Enquiry No. 110

Mass spectroscopy systems

MultiProbe

From Packard

Automates solid-phase extraction (SPE) in 96-well microplate formats for high-throughput bioanalysis using LC-MS-MS

This extraction system consists of a variety of vacuum manifolds and SPE blocks in a standard 96-well format. It can be mounted directly on the MultiProbe deck. The software for this system enables control of a vacuum system, allowing automation of all liquid handling steps, while minimizing user intervention. All liquid handling steps, including the dispensing of solvents, samples and internal standards, can be carried out by the system. Operating in the multi-tipped mode, as well as vacuum steps required to prime, load, wash and elute the blocks, the MultiProbe provides a high-throughput, front-end to mass spectrometry analyses such as those often performed in pharmaceutical biometabolism studies.

Reader Enquiry No. 111

HiRes FT-MS system

From IonSpec/GSG

Ultra-high resolution FT-MS for ESI and MALDI analysis of biomolecules

The company has launched its HiRes Fourier transform mass spectroscopy FT-IR system with electrospray ionization (ESI) and matrix-assisted, laser desorption ionization (MALDI) source options. The instrument uses a Fourier transform detection method and a quadrupole ion guide to provide the improved mass resolving power (stated to be 100× greater than quadrupole). It is said to be able to resolve naturally occurring isotopic forms of molecules and as a consequence directly assigns charge states, allowing molecular ion masses to be readily and accurately measured. Fragmentation of molecules can also be carried out, and from the charge-state calculation a deconvoluted spectrum is rapidly generated to assist in detailed structural recognition. The external ion-source design and Pentium-based software are designed to create a sensitive, reliable and easy-to-use mass spectroscopy technique. FT-MS should prove useful for MS analyses in molecular biology, biochemistry and their associated biotechnologies.

Reader Enquiry No. 112

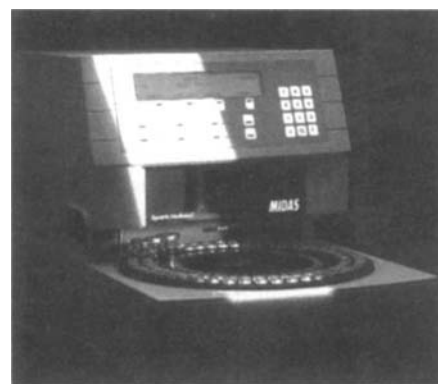
Putting colour in chromatography

Midas autosampler literature

From Jones Chromatography

A four-page brochure introducing the Midas HPLC autosampler

Manufactured by Spark Holland, Midas is designed to increase sample throughput, comply with good laboratory practice direc-



Spark Holland's Midas autosampler

tives, control HPLC temperatures, utilize high-resolution syringe control and communicate with external computers using self-guiding software. Additionally, the brochure illustrates how the unit employs pressure-assisted sample aspiration with three discrete injection modes: full-loop filling, partial-loop filling and microlitre pick up. The literature also describes the side-port, spring-loaded needle design, which assures that needles will aspirate entire sample volumes without becoming blocked by septum particles.

Reader Enquiry No. 113

PL-EMD 960 detector

From Polymer Laboratories

An application note on the evaporative, light-scattering detector

This application note covers the use of this detector as a high-sensitivity concentration detector, which can be used in HPLC, GPC and HTGPC, especially for samples that may contain compounds without chromophores. This literature includes the principle of operation for the detector and offers direct comparisons with refractive-index and ultraviolet detection, as well as product specifications. Applications showing chromatograms and conditions illustrate the suitability of the detector across a wide variety of application areas, including carbohydrates, peptides, pharmaceuticals, surfactants, triglycerides and vitamins, together with polyolefins at high temperature and orthogonal chromatography.

Reader Enquiry No. 114

Chrom Perfect Enterprise

From Russel Mainstream Software

A client/server chromatography data system run with a Windows NT user interface

Developed by Justice Innovations, this is a multi-user, client-server chromatography data system developed for laboratory and site-wide applications running Windows NT. System features include centralized administration, network security, and good laboratory practice procedures, including a

complete logbook and audit trail, validation, customized report generation, automatic quality checking and post-run statistical analysis. The system allows the analyst to view real-time instrument status from any terminal on the network or through a modem from a remote computer. Integral to the system design is the ability to acquire data using Justice Innovations' high-resolution A/D boards, PE Nelson 700 or 900 series interface or directly from HP5890 GCs. Fully compatible with single-user versions of Chrom Perfect, it provides an easy upgrade path without loss of historical data or major retraining costs. System configurations are stated to support chromatographs from any vendor.

Reader Enquiry No. 115

9001 gas chromatograph

From Finnigan

A report that reviews residual solvent analysis in pharmaceuticals

This technical report entitled *Residual solvents in drugs by static headspace analysis* highlights the analysis of organic volatile impurities in oral medications. A Finnigan 9001 GC was configured with a pre-column 'Y' splitter and two dissimilar megabore capillary columns for confirmational analysis on a flame-ionization and a tandem photo-ionization/flame ionization detector. Samples of ascorbic acid, magnesium stearate and acetaminophen were injected by a headspace autosampler using a gas-tight syringe. The MDLs for USP 467 method IV were met by the elimination of active sites usually found in autosampler designs that use transfer lines and valves, including methylene valves. Minimum detection levels are charted for five solvents including methylene chloride, chloroform, benzene, trichloroethene and 1,4-dioxane.

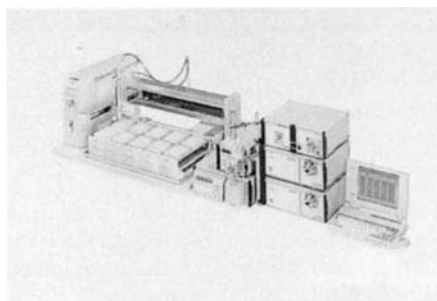
Reader Enquiry No. 116

HPLC combinatorial chromatography

From Gilson

A combinatorial chromatography system with a ten microplate capacity

To satisfy the demand for high throughput, Gilson has expanded the capacity of its HPLC combinatorial chromatography system. The system now accepts up to ten standard or deep-well microplates. Other vessel options are available for processes requiring purification or analysis of larger volumes. Users can choose from arrays, which include hundreds of 13-mm × 100-mm test tubes or 2-ml vials. Large-volume 185-ml bottles are also available. The system is also said to offer a high level of automation, allowing unattended operation. If reanalysis is required, the system can be set to reinject collected fractions automatically once purification has been com-



Gilson's combinatorial chemistry system

pleted. No manual intervention is required to replumb or reconfigure the system. The system can automatically switch between preparative and analytical volumes. Another key feature of the system is the graphic sample tracking software that automatically matches injections, chromatograms and fractions. The software eliminates the need to count event marks and should thus reduce error. The system also utilizes a dual-function instrument for fraction collection and injection, enabling samples to remain in the exact same position throughout the process.

Reader Enquiry No. 117

Star software

From Varian

Allows the automated production of customized chromatography reports

Varian's new Star custom report writer software is designed to improve laboratory productivity and decreases errors by eliminating the cumbersome process of transcribing detailed chromatographic results into a report format. This new tool, used with Varian's Star chromatography workstation version 4.5, allows chromatographers to customize reports with just a few clicks of the mouse. Designed for ease of use and learning, this program allows quick installation and should minimize training in automating data acquisition, analysis and reporting of analytical samples. Users can develop custom report templates for each type of sample and standard. The direct visual layout of the report templates enables on-screen editing of the report layout and design. Multiple chromatograms can be arranged side-by-side or overlaid on a single page report. As the custom report writer is integrated into the workstation method editor, each detector channel used in a method can have its own custom template. Custom templates help standardize the production of reports containing graphics images, user-selected results fields with complete text formatting, chromatograms, overlaid chromatograms, graphic images of reference chromatograms, company logos and addresses, and the like.

Reader Enquiry No. 118

Biosensorship

Sensor Chip NTA

From Pharmacia Biosensor

A new sensor chip for BIAcore biomolecular interaction analysis instruments

The new chip binds histidine-tagged (His-tag) molecules through chelated nickel for subsequent analysis of analyte binding. Immobilization of ligands using histidine tags provides a convenient interface between biomolecular interaction analysis and laboratory techniques that rely on His-tags, for example chelating chromatography. Immobilization via a His-tag also has the advantage of orienting the ligand molecules in a homogeneous way, allowing the immobilization to be carried out without significantly changing the pH or ionic strength during the coupling procedure.

Reader Enquiry No. 119

IASys

From Affinity Sensors

Two new, derivatized biosensor surfaces for IASys interaction analysis systems

The chemistry and geometry of the two new biosensor surfaces, carboxylate and biotin, allow a wide range of ligands to be immobilized onto the cuvette surface for rapid, high-sensitivity analysis of interaction kinetics in real time. The biotin surface has the ability to capture biotinylated lipid bilayers, which provides a more biologically relevant model for the study of lipid membrane binding. The new surfaces complement and expand the application flexibility offered by the carboxymethyl-dextran and amino cuvette surfaces. All cuvette surfaces are discussed in detail in a series of application notes available from the company.

Reader Enquiry No. 120

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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